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pages 2085-2093, see the whole article

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EP 0 643 767 B1

Description

Background of the Invention

5 This invention relates to the subject matter of the claims.

Mammalian bone tissue is known to contain one or more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

10 The developmental cascade of bone differentiation consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo. Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

15 The potential utility of these proteins has been recognized widely. It is contemplated that the availability of the protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. ((1987) Proc. Natl. Acad. Sci. USA 84: 7109-7113). Urist et al. (1984) Proc. Soc. Exp. Biol. Med. 173: 194-199 disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

20 Urist et al. (1984) Proc. Natl. Acad. Sci. USA 81: 371-375 disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors reported that the protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract from bone results in ill-defined and impure preparations.

25 European Patent Application Serial No. 148,155, published October 7, 1985, purports to disclose osteogenic proteins derived from bovine, porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into animals.

30 International Application No. PCT/087/01537, published January 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative "bone inductive factors" produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenic proteins, bone induction was not demonstrated, suggesting that the recombinant proteins are not osteogenic. The same group reported subsequently (Science, 242:1528, Dec, 1988) that three of the four factors induce cartilage formation, and postulate that bone formation activity "is due to a mixture of regulatory molecules" and that "bone formation is most likely controlled ... by the interaction of these molecules." Again, no bone induction was attributed to the products of expression of the cDNAs. See also Urist et al., EP0,212,474 entitled Bone Morphogenic Agents.

35 Wang et al. (1988) Proc. Nat. Acad. Sci. USA 85: 9484-9488 discloses the purification of a bovine bone morphogenic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

40 Wang et al. (1990) Proc. Nat. Acad. Sci. USA 87: 2220-2227 describes the expression and partial purification of one of the cDNA sequences described in PCT 87/01537. Consistent cartilage and/or bone formation with their protein requires a minimum of 600 ng of 50% pure material.

45 International Application No. PCT/89/04458 published April 19, 1990 (Int. Pub. No. W090/003733), describes the purification and analysis of a family of osteogenic factors called "P3 OF 31-34". The protein family contains at least

four proteins, which are characterized by peptide fragment sequences. The impure mixture P3 OF 31-34 is assayed for osteogenic activity. The activity of the individual proteins is neither assessed nor discussed.

Described herein are polypeptide chains useful as subunits of dimeric osteogenic proteins capable of endochondral bone formation in allogenic and xenogenic implants in mammals, including humans. Also described are genes encoding these polypeptide chains and methods for the production of osteogenic proteins comprising these polypeptide chains using recombinant DNA techniques, as well as antibodies capable of binding specifically to these proteins.

These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

Summary of the Invention

This invention concerns the subject matter of the claims, and provides novel polypeptide chains useful as either one or both subunits of dimeric osteogenic proteins which, when implanted in a mammalian body in association with a matrix, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation.

A key to these developments was the elucidation of amino acid sequence and structure data of native bovine osteogenic protein. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from bovine bone having a half-maximum bone forming activity of about 0.8 to 1.0 ng per mg of implant. The availability of the material enabled the inventors to elucidate key structural details of the protein necessary to achieve bone formation. Knowledge of the protein's amino acid sequence and other structural features enabled the identification and cloning of native genes in the human genome.

Consensus DNA sequences based on partial sequence data and observed homologies with regulatory proteins disclosed in the literature were used as probes for extracting genes encoding osteogenic protein from human genomic and cDNA libraries. One of the consensus sequences was used to isolate a previously unidentified gene which, when expressed, encoded a protein comprising a region capable of inducing endochondral bone formation when properly modified, incorporated in a suitable matrix, and implanted as disclosed herein. The gene, called "hOP1" or "OP-1" (human OP-1), is described in greater detail in copending U.S. 422,699, the disclosure of which is herein incorporated by reference.

In its native form, hOP1 expression yields an immature translation product ("hOP1-PP", where "PP" refers to "pre-pro form") of about 400 amino acids that subsequently is processed to yield a mature sequence of 139 amino acids ("OP1-18"). The active region (functional domain) of the protein comprises the C-terminal 97 amino acids of the hOP1 sequence ("OPS"). A long active sequence is OP7 (comprising the C-terminal 102 amino acids).

Further probing of mammalian cDNA libraries (human and mouse) with sequences specific to hOP1 also has identified novel OP1-like sequences herein referred to as "OP2" ("hOP2" or "mOP2"). The OP2 proteins share significant amino acid sequence homology, approximately 74%, with the active region of the OP1 proteins (e.g., OP7), and less homology with the intact mature form (e.g., OP1-18, 58% amino acid homology).

The amino acid sequence of the osteogenic proteins disclosed herein also share significant homology with various of the regulatory proteins on which the consensus probe was modeled. In particular, the proteins share significant homology in their C-terminal sequences, which comprise the active region of the osteogenic proteins. (Compare, for example, OP7 with DPP from *Drosophila* and Vgl from *Xenopus*. See, for example, U.S. Pat. No. 5,011,691). In addition, these proteins share a conserved six or seven cysteine skeleton in this region (e.g., the linear arrangement of these C-terminal cysteine residues is conserved in the different proteins.) See, for example, OP7, whose sequence defines the seven cysteine skeleton, or OPS, whose sequence defines the six cysteine skeleton. The OP2 proteins also contain an additional cysteine residue within this region.

Thus, in one preferred aspect, the invention comprises osteogenic proteins comprising a polypeptide chain comprising an amino acid sequence described by Seq. ID No. 3 or 5 such that a dimeric protein comprising this polypeptide chain has a conformation capable of inducing endochondral bone formation when implanted in a mammal in association with a suitable matrix. Useful proteins include the full-length protein and proteins comprising the functional domain described by the C-terminal.

In addition, the invention is not limited to these specific constructs. Thus, the osteogenic proteins of this invention comprising any of these polypeptide chains may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology which may be naturally occurring or biosynthetically derived, and active truncated forms of the native amino acid sequence, produced by expression of recombinant DNA in procaryotic or eucaryotic host cells. Active squances useful as osteogenic proteins of this invention are envisioned to include proteins capable of inducing endochondral bone formation when implanted in a mammal in association wiht a matrix and having at least a 70% sequence homology, preferably at least 80%, with the amino acid sequence of OPS.

The novel polypeptide chains and the osteogenic proteins they comprise can be expressed from intact or truncated

cDNA or from synthetic DNAs in procaryotic or eucaryotic host cells, and then purified, cleaved, refolded, dimerized, and implanted in experimental animals. Currently preferred host cells include *E.coli* or mammalian cells, such as CHO, COS or 35C cells. The osteogenic protein of the invention may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated forms of native or biosynthetic proteins, produced by expression of recombinant DNA in host cells.

Thus, in view of this disclosure, skilled genetic engineers can isolate genes from cDNA or genomic libraries of various different species which encode appropriate amino acid sequences, or construct DNAs from oligonucleotides, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active proteins capable of inducing bone formation in mammals including humans. In view of this disclosure, those skilled in the art, using standard immunology techniques also may create antibodies capable of binding specifically to the osteogenic proteins disclosed herein, including fragments thereof.

The osteogenic proteins are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles of porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range of 70 - 850 μ m, preferably 150 μ m - 420 μ m. It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable in vivo to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and specially treated particulate, protein extracted, demineralized, xenogenic bone. Optionally, such xenogenic bone powder matrices also may be treated with proteases such as trypsin and/or fibril modifying agents to increase the intraparticle intrusion volume and surface area. Useful agents include solvents such as dichloromethane, trichloroacetic acid, acetonitrile and acids such as trifluoroacetic acid and hydrogen fluoride. Alternatively, the matrix may be treated with a hot aqueous medium having a temperature within the range of about 37°C to 75°C, including a heated acidic aqueous medium. Other potentially useful matrix materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates.

The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) *Lancet* 1:959-963). The devices may be used to induce local endochondral bone formation in non-union fractures as demonstrated in animal tests, and in other clinical applications including dental and periodontal applications where bone formation is required. Another potential clinical application is in cartilage repair, for example, in the treatment of osteoarthritis.

Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1 compares the amino acid sequences of the mature mOP-2 and hOP-2 polypeptide chains: hOP2-A and mOP2-A; and

FIGURE 2 compares the amino acid sequences of the mature OP1 and OP2 polypeptide chains: OP1-18, mOP1-S, hOP2-A and mOP2-A.

Description

Purification protocols first were developed which enabled isolation of the osteogenic protein present in crude protein extracts from mammalian bone. (See PCT WO 89/09787, published 19-OCT-89, and U.S. Serial No. 179,406 filed April 8, 1988, now U.S. Patent No. 4,968,950). The development of the procedure, coupled with the availability of fresh calf bone, enabled isolation of substantially pure bovine osteogenic protein (bOP). bOP was characterized significantly; its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat were demonstrated and studied; it was shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone extracts. This dose dependent and highly specific activity was present whether or not the protein was glycosylated (see Sampath et al., (1990) *J. Biol. Chem.* 265: 13198-13205). Sequence data obtained from the bovine materials suggested probe designs which were used to isolate human genes. The OP human counterpart proteins have now been expressed and extensively characterized.

These discoveries enabled preparation of DNAs encoding totally novel, non-native protein constructs which individually as homodimers and combined with other species as heterodimers are capable of producing true endochondral

bone (see PCT WO 09788, published 19-OCT-89, and US Serial No. 315,342, filed 23-FEB-89, now U.S. Patent No. 5,011,691). They also permitted expression of the natural material, truncated forms, muteins, analogs, fusion proteins, and various other variants and constructs, from cDNAs and genomic DNAs retrieved from natural sources or from synthetic DNA produced using the techniques disclosed herein and using automated, commercially available equipment. The DNAs may be expressed using well established molecular biology and recombinant DNA techniques in procaryotic or eucaryotic host cells, and may be oxidized and refolded in vitro if necessary, to produce biologically active protein.

One of the DNA sequences isolated from human genomic and cDNA libraries encoded a previously unidentified gene, referred to herein as OP1. The protein encoded by the isolated DNA was identified originally by amino acid homology with proteins in the TGF- β family. Consensus splice signals were found where amino acid homologies ended, designating exon-intron boundaries. Three exons were combined to obtain a functional TGF- β -like domain containing seven cysteines. (See, for example, U.S. Patent No. 5,011,691, or Ozkaynak, E. et al., (1990) EMBO, 9: 2085-2093).

The full-length cDNA sequence for hOP1, and its encoded "prepro" form "hOP1-PP," which includes an N-terminal signal peptide sequence, are disclosed in Seq. ID No. 1 (residues 1-431). The mature form of the hOP1 protein expressed in mammalian cells, "OP1-18", is described by amino acid residues 293-431 of Seq. ID No. 1. The full length form of hOP1, as well as various truncated forms of the gene, and fused genes, have been expressed in E. coli and numerous mammalian cells (see, for example, published PCT application WO 91/05802, published 2-MAY-91) and all have been shown to have osteogenic activity when implanted in a mammal in association with a suitable matrix.

Given the foregoing amino acid and DNA sequence information, various nucleic acids (RNAs and DNAs) can be constructed which encode at least the active region of the hOP1 protein (e.g., OPS or OP7) and various analogs thereof (including allelic and species variants and those containing genetically engineered mutations), as well as fusion proteins, truncated forms of the mature proteins, and similar constructs. Moreover, DNA hybridization probes can be constructed from fragments of the hOP1 DNA or designed de novo based on the hOP1 DNA or amino acid sequence. These probes then can be used to screen different genomic and cDNA libraries to identify additional osteogenic proteins.

The DNAs can be produced by those skilled in the art using well known DNA manipulation techniques involving genomic and cDNA isolation, construction of synthetic DNA from synthesized oligonucleotides, and cassette mutagenesis techniques. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer. The DNA then may be electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purified by PAGE.

DNAs used as hybridization probes may be labelled (e.g., as with a radioisotope, by nick-translation) and used to identify clones in a given library containing DNA to which the probe hybridizes, following techniques well known in the art. The libraries may be obtained commercially or they may constructed de novo using conventional molecular biology techniques. Further information on DNA library construction and hybridization techniques can be found in numerous texts known to those skilled in the art. See, for example, F.M. Ausubel, ed., Current Protocols in Molecular Biology, Vol. 1, (1989). In particular, see unit 5, "Construction of Recombinant DNA Libraries" and Unit 6, "Screening of Recombinant Libraries."

The DNA from appropriately identified clones then can be isolated, subcloned (preferably into an expression vector), and sequenced. Plasmids containing sequences of interest then can be transfected into an appropriate host cell for protein expression and further characterization. The host may be a procaryotic or eucaryotic cell since the former's inability to glycosylate protein will not destroy the protein's osteogenic activity. Useful host cells include E. coli, Saccharomyces, the insect/baculovirus cell system, myeloma cells, and various mammalian cells. The vector additionally may encode various sequences to promote correct expression of the recombinant protein, including transcription promoter and termination sequences, enhancer sequences, preferred ribosome binding site sequences, preferred mRNA leader sequences, preferred signal sequences for protein secretion, and the like. The DNA sequence encoding the gene of interest also may be manipulated to remove potentially inhibiting sequences or to minimize unwanted secondary structure formation. The recombinant osteogenic protein also may be expressed as a fusion protein. After being translated, the protein may be purified from the cells themselves or recovered from the culture medium. All biologically active protein forms comprise dimeric species joined by disulfide bonds or otherwise associated, produced by oxidizing and refolding one or more of the various recombinant polypeptide chains within an appropriate eucaryotic cell or in vitro after expression of individual subunits. A detailed description of osteogenic protein expressed from recombinant DNA in E. coli is disclosed in U.S. Serial No. 660,162, filed 27-FEB-91, the disclosure of which incorporated by reference herein. A detailed description of osteogenic protein expressed from recombinant DNA in numerous different mammalian cells is disclosed in PCT WO91/05802, also incorporated herein by reference.

Finally, in view of the disclosure made herein, and using standard methodologies known in the art, persons skilled in the art can raise polyclonal and monoclonal antibodies against all or part of a polypeptide chain disclosed herein, such that the antibodies are capable of binding specifically to an epitope on the polypeptide chain. Useful protocols

can be found in, for example, Molecular Cloning-A Laboratory Manual (Sambrook et al. eds., Cold Spring Harbor Press 2nd ed., 1989). See Book 3, Section 18.

Exemplification

In an effort to identify additional DNA sequences encoding osteogenic proteins, a hybridization probe specific to the C-terminus of the DNA of mature OP-1 was prepared using a *Stu*I-EcoR1 digest fragment of OP-1 (base pairs 1034-1354 in Sequence ID No. 1), and labelled with ³²P by nick translation, as described in the art. As disclosed supra, the OP1 C-terminus encodes a key functional domain, e.g., the "active region" for osteogenic activity. The C-terminus also is the region of the protein whose amino acid sequence shares specific amino acid sequence homology with particular proteins in the TGF- β super-family of regulatory proteins, and which includes the conserved cysteine skeleton.

Approximately 7×10^5 phages of an oligo(dT) primed 17.5 days p.c. mouse embryo 5' stretch cDNA (gt10) library (Clontech, Inc., Palo Alto, CA) was screened with the labelled probe. The screen was performed using the following stringent hybridization conditions: 40% formamide, 5 x SSPE, 5 x Denhart's solution, 0.1% SDS, at 37°C overnight, and washing in 0.1 x SSPE, 0.1% SDS at 50°C.

Five recombinant phages were purified over three rounds of screening. Phage DNA was prepared from all five phages, subjected to an EcoR1 digest, subcloned into the EcoR1 site of a common pUC-type plasmid modified to allow single strand sequencing, and sequenced using means well known in the art.

Two different DNAs were identified by this procedure. One DNA, referred to herein as mOP1, has substantial homology to the mature form of OP1 (about 98%). A second DNA, encoding the C-terminus of a related gene and referred to herein as mOP2, also was identified by this procedure. The N-terminus of the gene encoding mOP2 was identified subsequently by screening a second mouse cDNA library (Mouse PCC4 cDNA (ZAP) library, Stratagene, Inc., La Jolla, CA).

Mouse OP2 (mOP2) protein shares significant amino acid sequence homology with the amino acid sequence of the hOP1 active region, e.g., OPS or OP7, about 74% homology, and less homology with the intact mature form, e.g., OP1-18, about 58% homology. The cDNA sequence, and the encoded amino acid sequence, for the full length mOP-2 protein is depicted in Sequence ID No. 3. The full-length form of the protein is referred to as the prepro form of mOP-2 ("mOP2-PP"), and includes a signal peptide sequence at its N-terminus. The amino acid sequence Leu-Ala-Leu-Cys-Ala-Leu (amino acid residues 13-18 of Sequence ID No. 3) is believed to constitute the cleavage site for the removal of the signal peptide sequence, leaving an intermediate form of the protein, the "pro" form, to be secreted from the expressing cell. The amino acid sequence Arg-Ala-Pro-Arg-Ala (amino acid residues 255-259 of Sequence ID No. 3) is believed to constitute the cleavage site that produces the mature form of the protein, herein referred to as "mOP2-A", and described by residues 259-397 of Seq. ID No. 3. Residues 301-397 of Seq. ID No. 3 correspond to the region defining the conserved six cysteine skeleton. Residues 296-397 of Seq. ID No. 3 correspond to the region defining the conserved seven cysteine skeleton.

Using a probe prepared from the pro region of mOP2 (an EcoR1-BamH1 digest fragment, bp 467-771 of Sequence ID No. 3), a human hippocampus library was screened (human hippocampus cDNA lambda (ZAP II library Stratagene, Inc., La Jolla, CA) following essentially the same procedure as for the mouse library screens. The procedure identified the N-terminus of a novel DNA encoding an amino acid sequence having substantial homology with mOP2. The C-terminus of the gene subsequently was identified by probing a human genomic library (in lambda phage EMBL-3, Clontech, Inc., Palo Alto, CA) with a labelled fragment from the novel human DNA in hand. The novel polypeptide chain encoded by this DNA is referred to herein as hOP2 protein, and shares almost complete amino acid identity (about 92% amino acid sequence homology) with mOP2-A (see Fig. 1 and infra).

The cDNA sequence, and the encoded amino acid sequence, for the prepro form of hOP2, "hOP2-PP", is described in Sequence ID No. 5. This full-length form of the protein also includes a signal peptide sequence at its N-terminus. The amino acid sequence Leu-Ala-Leu-Cys-Ala-Leu (amino acid residues 13-18 of Sequence ID No. 5) is believed to constitute the cleavage site for the removal of the signal peptide sequence, leaving an intermediate form of the protein, the "pro" form, to be secreted from the expressing cell. The amino acid sequence Arg-Thr-Pro-Arg-Ala (amino acid residues 257-261 of Sequence ID No. 5) is believed to constitute the cleavage site that produces what is believed to be the mature form of the protein, herein referred to as hOP2-A" and described by residues 261-399 of Seq. ID No. 5.

Additional mature species of hOP2 thought to be active include truncated sequences, "hOP2-P" (described by residues 264-399 of Seq. ID No. 5) and "hOP2-R" (described by residues 267-399 of Seq. ID No. 5), and a slightly longer sequence ("hOP2-S", described by residues 240-399 of Seq. ID No. 5). Residues 303-399 of Seq. ID No. 5 correspond to the region defining the conserved six cysteine skeleton. Residues 298-399 of Seq. ID No. 5 correspond to the region defining the conserved seven cysteine skeleton.

It should be noted that the nucleic acid sequence encoding the N-terminus of the prepro form of both mOP2 and hOP2 is rich in guanine and cytosine base pairs. As will be appreciated by those skilled in the art, sequencing such a "G-C rich" region can be problematic, due to stutter and/or band compression. Accordingly, the possibility of sequenc-

ing errors in this region can not be ruled out. However, the definitive amino acid sequence for these and other, similarly identified proteins can be determined readily by expressing the protein from recombinant DNA using, for example, any of the means disclosed herein, and sequencing the polypeptide chain by conventional peptide sequencing methods well known in the art.

Figure 1 compares the amino acid sequences of mature mOP2 and hOP2. Identity is indicated by three dots (...) in the mOP2 sequence. As is evident from the figure, the amino acid sequence homology between the mature forms of these two proteins is substantial (92% homology between the mature sequences, about 95% homology within the C-terminal active region (e.g., residues 38-139 or 42-139 of Fig. 1.)

Fig. 2 compares the amino acid sequences for the mature forms of all four species of OP1 and OP2 proteins. Here again, identity is indicated by three dots (...). Like the mOP2 protein, the hOP2 protein shares significant homology (about 74%) with the amino acid sequence defining the OP1 active region (OPS or OP7, residues 43-139 and 38-139, respectively, in Fig. 2), and less homology with OP1-18 (about 58% homology). Both OP2 proteins share the conserved seven cysteine skeleton seen in the OP1 proteins. In addition, the OP2 proteins comprise an eighth cysteine residue within this region (see position 78 in FIG. 2).

A preferred generic amino acid sequence useful as a subunit of a dimeric osteogenic protein capable of inducing endochondral bone or cartilage formation when implanted in a mammal in association with a matrix, and which incorporates the maximum homology between the identified OP1 and OP2 proteins, can be described by the sequence referred to herein as "OPX", described below and in Seq. No.7.

	Cys	Xaa	Xaa	His	Glu	Leu	Tyr	Val	Xaa	Phe
	1				5					10
	Xaa	Asp	Leu	Gly	Trp	Xaa	Asp	Trp	Xaa	Ile
					15					20
	Ala	Pro	Xaa	Gly	Tyr	Xaa	Ala	Tyr	Tyr	Cys
					25					30
	Glu	Gly	Glu	Cys	Xaa	Phe	Pro	Leu	Xaa	Ser
					35					40
	Xaa	Met	Asn	Ala	Thr	Asn	His	Ala	Ile	Xaa
					45					50
	Gln	Xaa	Leu	Val	His	Xaa	Xaa	Xaa	Pro	Xaa
					55					60
	Xaa	Val	Pro	Lys	Xaa	Cys	Cys	Ala	Pro	Thr
					65					70
	Xaa	Leu	Xaa	Ala	Xaa	Ser	Val	Leu	Tyr	Xaa
					75					80
	Asp	Xaa	Ser	Xaa	Asn	Val	Xaa	Leu	Xaa	Lys
					85					90
	Xaa	Arg	Asn	Met	Val	Val	Xaa	Ala	Cys	Gly
					95					100
	Cys	His,								

and wherein Xaa at res. 2 = (Lys or Arg); Xaa at res. 3 = (Lys or Arg); Xaa at res. 9 = (Ser or Arg); Xaa at res. 11 = (Arg or Gln); Xaa at res. 16 = (Gln or Leu); Xaa at res. 19 = (Ile or Val); Xaa at res. 23 = (Glu or Gln); Xaa at res. 26 = (Ala or Ser); Xaa at res. 35 = (Ala or Ser); Xaa at res. 39 = (Asn or Asp); Xaa at res. 41 = (Tyr or Cys); Xaa at res. 50 = (Val or Leu); Xaa at res. 52 = (Ser or Thr); Xaa at res. 56 = (Phe or Leu); Xaa at res. 57 = (Ile or Met); Xaa at res. 58 = (Asn or Lys); Xaa at res. 60 = (Glu, Asp or Asn); Xaa at res. 61 = (Thr, Ala or Val); Xaa at res. 65 = (Pro or Ala); Xaa at res. 71 = (Gln or Lys); Xaa at res. 73 = (Asn or Ser); Xaa at res. 75 = (Ile or Thr); Xaa at res. 80 = (Phe or Tyr);

Xaa at res. 82 = (Asp or Ser); Xaa at res. 84 = (Ser or Asn); Xaa at res. 87 = (Ile or Asp); Xaa at res. 89 = (Lys or Arg); Xaa at res. 91 = (Tyr, Ala or His); and Xaa at res. 97 = (Arg or Lys).

The high degree of homology exhibited between the various OP1 and OP2 proteins suggests that the novel osteogenic proteins identified herein will purify essentially as OP1 does, or with only minor modifications of the protocols disclosed for OP1. Similarly, the purified mOP1, mOP2, and hOP2 proteins are predicted to have an apparent molecular weight of about 18 kDa as reduced single subunits, and an apparent molecular weight of about 36 kDa as oxidized dimers, as determined by comparison with molecular weight standards on an SDS-polyacrylamide electrophoresis gel. Unglycosylated dimers (e.g., proteins produced by recombinant expression in *E. coli*) are predicted to have an apparent molecular weight of about 27 kDa. There appears to be one potential N glycosylation site in the mature forms of the mOP2 and hOP2 proteins.

The identification of osteogenic proteins having an active region comprising eight cysteine residues also allows one to construct osteogenic polypeptide chains patterned after either of the following template amino acid sequences, or to identify additional osteogenic proteins having this sequence. The template sequences contemplated are "OPX-7C", comprising the conserved six cysteine skeleton plus the additional cysteine residue identified in the OP2 proteins, and "OPX-8C", comprising the conserved seven cysteine skeleton plus the additional cysteine residue identified in the OP2 proteins. The OPX-7C and OPX-8C sequences are described below and in Seq. ID Nos. 8 and 9, respectively. Each Xaa in these template sequences independently represents one of the 20 naturally-occurring L-isomer, α -amino acids, or a derivative thereof. Biosynthetic constructs patterned after this template readily are constructed using conventional DNA synthesis or peptide synthesis techniques well known in the art. Once constructed, osteogenic proteins comprising these polypeptide chains can be tested as disclosed herein.

"OPX-7C" (Sequence ID No. 8) :

```

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
  1                      5                      10
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                      15                      20
Xaa Xaa Cys Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa
          25                      30
Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa

          35                      40
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
  45                      50                      55
Xaa Xaa Xaa Xaa Xaa Cys Cys Xaa Xaa Xaa Xaa
          60                      65
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys Xaa
          90                      95

```

"OPX-8C" (Sequence ID No. 9 comprising additional five residues at the N-terminus, including a conserved cysteine residue):

Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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 5 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 15 20
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa
 25 30
 10 Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa
 35 40 45
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 15 50 55
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
 60 65
 20 Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 70 75
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 80 85
 25 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
 90 95
 Xaa Cys Xaa

100

35 MATRIX PREPARATION

A. General Consideration of Matrix Properties

The currently preferred carrier material is a xenogenic bone-derived particulate matrix treated as disclosed herein.
 40 This carrier may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., hydroxylapatite (HAP), collagen, tricalcium phosphate or polylactic acid, polyglycolic acid and various copolymers thereof.)

Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone;
 45 particles between 75 μ m and 420 μ m elicit the maximum response. Contamination of the matrix with bone mineral will inhibit bone formation. Most importantly, the procedures used to formulate OP onto the matrix are extremely sensitive to the physical and chemical state of both the osteogenic protein and the matrix.

The sequential cellular reactions in the interface of the bone matrix/osteogenic protein implants are complex. The multistep cascade includes: binding of fibrin and fibronectin to implanted matrix, chemotaxis of cells, proliferation of
 50 fibroblasts, differentiation into chondroblasts, cartilage formation, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible
 55 in vivo and preferably biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates in vivo. In bones, the dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

Matrix geometry, particle size, the presence of surface charge, and the degree of both intra-and-inter-particle

porosity are all important to successful matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue. Masticated muscle or other tissue may also be used. Large allogenic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particle and the dispersed osteogenic protein.

The preferred matrix material, prepared from xenogenic bone and treated as disclosed herein, produces an implantable material useful in a variety of clinical settings. In addition to its use as a matrix for bone formation in various orthopedic, periodontal, and reconstructive procedures, the matrix also may be used as a sustained release carrier, or as a collagenous coating for implants. The matrix may be shaped as desired in anticipation of surgery or shaped by the physician or technician during surgery. Thus, the material may be used for topical, subcutaneous, intraperitoneal, or intramuscular implants; it may be shaped to span a nonunion fracture or to fill a bone defect. In bone formation or conduction procedures, the material is slowly absorbed by the body and is replaced by bone in the shape of or very nearly the shape of the implant.

Various growth factors, hormones, enzymes, therapeutic compositions, antibiotics, and other body treating agents also may be absorbed onto the carrier material and will be released over time when implanted as the matrix material is slowly absorbed. Thus, various known growth factors such as EGF, PDGF, IGF, FGF, TGF- α , and TGF- β may be released in vivo. The material can be used to release chemotherapeutic agents, insulin, enzymes, or enzyme inhibitors.

B. Bone-Derived Matrices

1. Preparation of Demineralized Bone

Demineralized bone matrix, preferably bovine bone matrix, is prepared by previously published procedures (Sam-path and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595). Bovine diaphyseal bones (age 1-10 days) are obtained from a local slaughterhouse and used fresh. The bones are stripped of muscle and fat, cleaned of periosteum, demarrowed by pressure with cold water, dipped in cold absolute ethanol, and stored at -20°C. They are then dried and fragmented by crushing and pulverized in a large mill. Care is taken to prevent heating by using liquid nitrogen. The pulverized bone is milled to a particle size in the range of 70-850 μm , preferably 150-420 μm , and is defatted by two washes of approximately two hours duration with three volumes of chloroform and methanol (3:1). The particulate bone is then washed with one volume of absolute ethanol and dried over one volume of anhydrous ether yielding defatted bone powder. The defatted bone powder is then demineralized by four successive treatments with 10 volumes of 0.5 N HCl at 4°C for 40 min. Finally, neutralizing washes are done on the demineralized bone powder with a large volume of water.

2. Guanidine Extraction

Demineralized bone matrix thus prepared is extracted with 5 volumes of 4 M guanidine-HCl, 50mM Tris-HCl, pH 7.0 for 16 hr. at 4°C. The suspension is filtered. The insoluble material is collected and used to fabricate the matrix. The material is mostly collagenous in nature. It is devoid of osteogenic or chondrogenic activity.

3. Matrix Treatments

The major component of all bone matrices is Type-I collagen. In addition to collagen, demineralized bone extracted as disclosed above includes non-collagenous proteins which may account for 5% of its mass. In a xenogenic matrix, these noncollagenous components may present themselves as potent antigens, and may constitute immunogenic and/or inhibitory components. These components also may inhibit osteogenesis in allogenic implants by interfering with the developmental cascade of bone differentiation. It has been discovered that treatment of the matrix particles with a collagen fibril-modifying agent extracts potentially unwanted components from the matrix, and alters the surface structure of the matrix material. Useful agents include acids, organic solvents or heated aqueous media. Various treatments are described below. A detailed physical analysis of the effect these fibril-modifying agents have on demineralized, guanidine-extracted bone collagen particles is disclosed in PCT WO 90/10018, published 7-SEP-90.

After contact with the fibril-modifying agent, the treated matrix is washed to remove any extracted components, following a form of the procedure set forth below:

1. Suspend in TBS (Tris-buffered saline) 1g/200 ml and stir at 4°C for 2 hrs; or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS) or water and stir at room temperature (RT) for 30 minutes (sufficient time to neutralize

the pH);

2. Centrifuge and repeat wash step; and

3. Centrifuge; discard supernatant; water wash residue; and then lyophilize.

3.1 Acid Treatments

1. Trifluoroacetic acid.

Trifluoroacetic acid is a strong non-oxidizing acid that is a known swelling agent for proteins, and which modifies collagen fibrils.

Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are collected. These particles are extracted with various percentages (1.0% to 100%) of trifluoroacetic acid and water (v/v) at 0°C or room temperature for 1-2 hours with constant stirring. The treated matrix is filtered, lyophilized, or washed with water/salt and then lyophilized.

2. Hydrogen Fluoride.

Like trifluoroacetic acid, hydrogen fluoride is a strong acid and swelling agent, and also is capable of altering intraparticle surface structure. Hydrogen fluoride is also a known deglycosylating agent. As such, HF may function to increase the osteogenic activity of these matrices by removing the antigenic carbohydrate content of any glycoproteins still associated with the matrix after guanidine extraction.

Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are collected. The sample is dried in vacuo over P₂O₅, transferred to the reaction vessel and exposed to anhydrous hydrogen fluoride (10-20 ml/g of matrix) by distillation onto the sample at -70°C. The vessel is allowed to warm to 0°C and the reaction mixture is stirred at this temperature for 120 minutes. After evaporation of the hydrogen fluoride in vacuo, the residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid. Extent of deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with hydrogen fluoride, after washing the samples appropriately to remove non-covalently bound carbohydrates. SDS-extracted protein from HF-treated material is negative for carbohydrate as determined by Con A blotting.

The deglycosylated bone matrix is next washed twice in TBS (Tris-buffered saline) or UTBS, water-washed, and then lyophilized.

Other acid treatments are envisioned in addition to HF and TFA. TFA is a currently preferred acidifying reagent in these treatments because of its volatility. However, it is understood that other, potentially less caustic acids may be used, such as acetic or formic acid.

3.2 Solvent Treatment

1. Dichloromethane.

Dichloromethane (DCM) is an organic solvent capable of denaturing proteins without affecting their primary structure. This swelling agent is a common reagent in automated peptide synthesis, and is used in washing steps to remove components.

Bovine bone residue, prepared as described above, is sieved, and particles of the appropriate size are incubated in 100% DCM or, preferably, 99.9% DCM/0.1% TFA. The matrix is incubated with the swelling agent for one or two hours at 0°C or at room temperature. Alternatively, the matrix is treated with the agent at least three times with short washes (20 minutes each) with no incubation.

2. Acetonitrile.

Acetonitrile (ACN) is an organic solvent, capable of denaturing proteins without affecting their primary structure. It is a common reagent used in high-performance liquid chromatography, and is used to elute proteins from silica-based columns by perturbing hydrophobic interactions.

Bovine bone residue particles of the appropriate size, prepared as described above, are treated with 100% ACN (1.0 g/30 ml) or, preferably, 99.9% ACN/0.1% TFA at room temperature for 1-2 hours with constant stirring. The treated matrix is then water-washed, or washed with urea buffer, or 4 M NaCl and lyophilized. Alternatively, the ACN or ACN/TFA treated matrix may be lyophilized without wash.

3. Isopropanol.

Isopropanol is also an organic solvent capable of denaturing proteins without affecting their primary structure. It is a common reagent used to elute proteins from silica HPLC columns.

Bovine bone residue particles of the appropriate size prepared as described above are treated with 100% isopropanol (1.0 g/30 ml) or, preferably, in the presence of 0.1% TFA, at room temperature for 1-2 hours with constant stirring. The matrix is then water-washed or washed with urea buffer or 4 M NaCl before being lyophilized.

4. Chloroform

Chloroform also may be used to increase surface area of bone matrix like the reagents set forth above, either alone or acidified.

Treatment as set forth above is effective to assure that the material is free of pathogens prior to implantation.

3.3 Heat Treatment

The currently most preferred agent is a heated aqueous fibril-modifying medium such as water, to increase the matrix particle surface area and porosity. The currently most preferred aqueous medium is an acidic aqueous medium having a pH of less than about 4.5, e.g., within the range of pH 2 - pH 4, which may help to "swell" the collagen before heating. 0.1% acetic acid, which has a pH of about 3, currently is preferred. 0.1 M acetic acid also may be used.

Various amounts of delipidated, demineralized guanidine-extracted bone collagen are heated in the aqueous medium (1g matrix/30ml aqueous medium) under constant stirring in a water jacketed glass flask, and maintained at a given temperature for a predetermined period of time. Preferred treatment times are about one hour, although exposure times of between about 0.5 to two hours appear acceptable. The temperature employed is held constant at a temperature generally within the range of about 37°C to 75°C. The currently preferred heat treatment temperature is within the range of 45°C to 60°C.

After the heat treatment, the matrix is filtered, washed, lyophilized and used for implant. Where an acidic aqueous medium is used, the matrix also is preferably neutralized prior to washing and lyophilization. A currently preferred neutralization buffer is a 200mM sodium phosphate buffer, pH 7.0. To neutralize the matrix, the matrix preferably first is allowed to cool following thermal treatment, the acidic aqueous medium (e.g., 0.1% acetic acid) then is removed and replaced with the neutralization buffer and the matrix agitated for about 30 minutes. The neutralization buffer then may be removed and the matrix washed and lyophilized (see infra).

The matrix also may be treated to remove contaminating heavy metals, such as by exposing the matrix to a metal ion chelator. For example, following thermal treatment with 0.1% acetic acid, the matrix may be neutralized in a neutralization buffer containing EDTA (sodium ethylenediaminetetraacetic acid), e.g., 200 mM sodium phosphate, 5mM EDTA, pH 7.0. 5 mM EDTA provides about a 100-fold molar excess of chelator to residual heavy metals present in the most contaminated matrix tested to date. Subsequent washing of the matrix following neutralization appears to remove the bulk of the EDTA. EDTA treatment of matrix particles reduces the residual heavy metal content of all metals tested (Sb, As, Be, Cd, Cr, Cu, Co, Pb, Hg, Ni, Se, Ag, Zn, Tl) to less than about 1 ppm. Bioassays with EDTA-treated matrices indicate that treatment with the metal ion chelator does not inhibit bone inducing activity.

The collagen matrix materials preferably take the form of a fine powder, insoluble in water, comprising nonadherent particles. It may be used simply by packing into the volume where new bone growth or sustained release is desired, held in place by surrounding tissue. Alternatively, the powder may be encapsulated in, e.g., a gelatin or polylactic acid coating, which is adsorbed readily by the body. The powder may be shaped to a volume of given dimensions and held in that shape by interadhering the particles using, for example, soluble, species-biocompatible collagen. The material may also be produced in sheet, rod, bead, or other macroscopic shapes.

Demineralized rat bone matrix used as an allogenic matrix in certain of the experiments disclosed herein, is prepared from several of the dehydrated diaphyseal shafts of rat femur and tibia as described herein to produce a bone particle size which passes through a 420 μ m sieve. The bone particles are subjected to dissociative extraction with 4 M guanidine-HCl. Such treatment results in a complete loss of the inherent ability of the bone matrix to induce endochondral bone differentiation. The remaining insoluble material is used to fabricate the matrix. The material is mostly collagenous in nature, and upon implantation, does not induce cartilage and bone. All new preparations are tested for mineral content and osteogenic activity before use. The total loss of biological activity of bone matrix is restored when an active osteoinductive protein fraction or a pure osteoinductive protein preparation is reconstituted with the biologically inactive insoluble collagenous matrix.

FABRICATION OF OSTEOGENIC DEVICE

The naturally sourced and recombinant protein as set forth above, and other constructs, can be combined and dispersed in a suitable matrix preparation using any of the methods described below. In general, 50-100 ng of active protein is combined with the inactive carrier matrix (e.g., 25 mg for rat bioassays). Greater amounts may be used for large implants.

1. Ethanol Precipitation

Matrix is added to osteogenic protein dissolved in guanidine-HCl. Samples are vortexed and incubated at a low temperature (e.g., 4°C). Samples are then further vortexed. Cold absolute ethanol (5 volumes) is added to the mixture which is then stirred and incubated, preferably for 30 minutes at -20°C. After centrifugation (microfuge, high speed) the supernatant is discarded. The reconstituted matrix is washed twice with cold concentrated ethanol in water (85% EtOH) and then lyophilized.

2. Acetonitrile Trifluoroacetic

Acid Lyophilization

In this procedure, osteogenic protein in an acetonitrile trifluoroacetic acid (ACN/TFA) solution is added to the carrier material. Samples are vigorously vortexed many times and then lyophilized. This method is currently preferred, and has been tested with osteogenic protein at varying concentrations and different levels of purity.

3. Urea Lyophilization

For those osteogenic proteins that are prepared in urea buffer, the protein is mixed with the matrix material, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

4. Buffered Saline Lyophilization

OP1 and OP2 preparations in physiological saline may also be vortexed with the matrix and lyophilized to produce osteogenically active material.

These procedures also can be used to adsorb other active therapeutic drugs, hormones, and various bioactive species to the matrix for sustained release purposes.

BIOASSAY

The functioning of the various proteins and devices of this invention can be evaluated with an in vivo bioassay. Studies in rats show the osteogenic effect in an appropriate matrix to be dependent on the dose of osteogenic protein dispersed in the matrix. No activity is observed if the matrix is implanted alone. In vivo bioassays performed in the rat model also have shown that demineralized, guanidine-extracted xenogenic bone matrix materials of the type described in the literature are ineffective as a carrier, fail to induce bone, and produce an inflammatory and immunological response when implanted unless treated as disclosed above. In certain species (e.g., monkey) allogenic matrix materials also apparently are ineffective as carriers. The following sets forth various procedures for preparing osteogenic devices from the proteins and matrix materials prepared as set forth above, and for evaluating their osteogenic utility.

A. Rat Model

1. Implantation

The bioassay for bone induction as described by Sampath and Reddi ((1983) Proc. Natl. Acad. Sci. USA 80 6591-6595), herein incorporated by reference, may be used to monitor endochondral bone differentiation activity. This assay consists of implanting test samples in subcutaneous sites in recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants were removed on day 12. The heterotopic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotopic sites. As disclosed herein, both allogenic (rat

bone matrix) and xenogenic (bovine bone matrix) implants were assayed.

2. Cellular Events

5 Successful implants exhibit a controlled progression through the stages of protein-induced endochondral bone development, including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of
10 the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

3. Histological Evaluation

15 Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in paraffin, and cut into 6-8 μ m sections. Staining with toluidine blue or hematoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve day implants are usually sufficient to determine whether the implants contain newly induced bone.

20 4. Biological Markers

Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology have little or no alkaline phosphatase activity under
25 these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation quickly after the implants are removed from the rat. Alternatively, the amount of bone formation can be determined by measuring the calcium content of the implant.

The invention may be embodied in other specific forms. The present embodiments are therefore to be considered
30 in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

35 (i) APPLICANT: OPPERMAN, HERNANN

OZKAYNAK, ENGIN
RUEGER, DAVID C.
40 KUBERASAMPATH, THANGAVEL

(ii) TITLE OF INVENTION: OSTEOGENIC DEVICES

(iii) NUMBER OF SEQUENCES: 9

45 (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: TESTA, HURVITZ & THIBEAULT
(B) STREET: 53 STATE STREET
50 (C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: U.S.A.
(F) ZIP: 02109

55 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: PITCHER, EDMUND R.
(B) REGISTRATION NUMBER: 27,829
(C) REFERENCE/DOCKET NUMBER: CRR056PC

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617/248-7000
(B) TELEFAX: 617/248-7100

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1822 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS
(F) TISSUE TYPE: HIPPOCAMPUS

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 49..1341
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"

/product= "hOP1-PP"
/evidence= EXPERIMENTAL
/standard_name= "hOP1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 431 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: protein

(ix) FEATURE:

55 (D) OTHER INFORMATION: /Product="h0P1-PP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 Met Phe Met Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Gly Gly
 85 90 95
 20 Gly Pro Gly Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser
 100 105 110
 Thr Gln Gly Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr
 115 120 125
 25 Asp Ala Asp Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys
 130 135 140
 Glu Phe Phe His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu
 145 150 155 160
 30 Ser Lys Ile Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile
 165 170 175
 Tyr Lys Asp Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Arg Ile
 180 185 190
 Ser Val Tyr Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu
 195 200 205
 40 Phe Leu Leu Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu
 210 215 220
 Val Phe Asp Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg
 225 230 235 240
 45 His Asn Leu Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser
 245 250 255
 50 Ile Asn Pro Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn

	260	265	270
5	Lys Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe 275 280 285		
	Arg Ser Ile Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser 290 295 300		
10	Lys Thr Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu 305 310 315 320		
	Asn Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr 325 330 335		
15	Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu 340 345 350		
	Gly Tyr Ala Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn 355 360 365		
20	Ser Tyr Met Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His 370 375 380		
	Phe Ile Asn Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln 385 390 395 400		
	Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile 405 410 415		
30	Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His 420 425 430		

(2) INFORMATION FOR SEQ ID NO:3:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1929 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

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(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 103..1293
 (D) OTHER INFORMATION: /function= "osteogenic protein"

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/product= "mOP2-PP"
 /note= "mOP2 cDNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

55

GAATTCGCT GCCAGGCACA GGTGCGCCGT CTGGTCCTCC CCGTCTGGCG TCAGCCGAGC

	CCGACCAGCT ACCAGTGGAT GCGCGCCGGC TGAAAGTCCG AG ATG GCT ATG CGT	114
	Met Ala Met Arg	
	1	
5		
	CCC GGG CCA CTC TGG CTA TTG GGC CTT GCT CTG TGC GCG CTG GGA GGC	162
	Pro Gly Pro Leu Trp Leu Leu Gly Leu Ala Leu Cys Ala Leu Gly Gly	
	5 10 15 20	
10		
	GGC CAC GGT CCC GGT CCC CCG CAC ACC TGT CCC CAG CGT CGC CTG GGA	210
	Gly His Gly Pro Gly Pro Pro His Thr Cys Pro Gln Arg Arg Leu Gly	
	25 30 35	
15		
	GCG CGC GAC CGG GAC ATG CAG CGT GAA ATC CTG CCG GTG CTC GGG CTA	258
	Ala Arg Asp Arg Asp Met Gln Arg Glu Ile Leu Pro Val Leu Gly Leu	
	40 45 50	
20		
	CCG GGA CGC CCC GAC CCC GTG CAC AAC CCG CCG CTG CCC GGC ACG CAG	306
	Pro Gly Arg Pro Asp Pro Val His Asn Pro Pro Leu Pro Gly Thr Gln	
	55 60 65	
25		
	CGT GCG CCC CTC TTC ATG TTG GAC CTA TAC CAC GCC ATG ACC GAT GAC	354
	Arg Ala Pro Leu Phe Met Leu Asp Leu Tyr His Ala Met Thr Asp Asp	
	70 75 80	
30		
	GAC GAC GGC GGG CCA CCA CAG GCT CAC TTA GGC CGT GCC GAC CTG GTC	402
	Asp Asp Gly Gly Pro Pro Gln Ala His Leu Gly Arg Ala Asp Leu Val	
	85 90 95 100	
35		
	ATG AGC TTC GTC AAC ATG GTG GAA CGC GAC CGT ACC CTG GGC TAC CAG	450
	Met Ser Phe Val Asn Met Val Glu Arg Asp Arg Thr Leu Gly Tyr Gln	
	105 110 115	
40		
	GAG CCA CAC TGG AAG GAA TTC CAC TTT GAC CTA ACC CAG ATC CCT GCT	498
	Glu Pro His Trp Lys Glu Phe His Phe Asp Leu Thr Gln Ile Pro Ala	
	120 125 130	
45		
	GGG GAG GCT GTC ACA GCT GCT GAG TTC CGG ATC TAC AAA GAA CCC AGC	546
	Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Glu Pro Ser	
	135 140 145	
50		
	ACC CAC CCG CTC AAC ACA ACC CTC CAC ATC AGC ATG TTC GAA GTG GTC	594
	Thr His Pro Leu Asn Thr Thr Leu His Ile Ser Met Phe Glu Val Val	
	150 155 160	
55		
	CAA GAG CAC TCC AAC AGG GAG TCT GAC TTG TTC TTT TTG GAT CTT CAG	642
	Gln Glu His Ser Asn Arg Glu Ser Asp Leu Phe Phe Leu Asp Leu Gln	
	165 170 175 180	
60		
	ACG CTC CGA TCT GGG GAC GAG GGC TGG CTG GTG CTG GAC ATC ACA GCA	690
	Thr Leu Arg Ser Gly Asp Glu Gly Trp Leu Val Leu Asp Ile Thr Ala	
	185 190 195	
65		
	GCC AGT GAC CGA TGG CTG CTG AAC CAT CAC AAG GAC CTG GGA CTC CGC	738

Ala	Ser	Asp	Arg	Trp	Leu	Leu	Asn	His	Lys	Asp	Leu	Gly	Leu	Arg	
		200					205					210			
CTC	TAT	GTG	GAA	ACC	GCG	GAT	GGG	CAC	AGC	ATG	GAT	CCT	GGC	CTG	GCT
Leu	Tyr	Val	Glu	Thr	Ala	Asp	Gly	His	Ser	Met	Asp	Pro	Gly	Leu	Ala
		215					220					225			
GGT	CTG	CTT	GGA	CGA	CAA	GCA	CCA	CGC	TCC	AGA	CAG	CCT	TTC	ATG	GTA
Gly	Leu	Leu	Gly	Arg	Gln	Ala	Pro	Arg	Ser	Arg	Gln	Pro	Phe	Met	Val
		230				235					240				
ACC	TTC	TTC	AGG	GCC	AGC	CAG	AGT	CCT	GTG	CGG	GCC	CCT	CGG	GCA	GCG
Thr	Phe	Phe	Arg	Ala	Ser	Gln	Ser	Pro	Val	Arg	Ala	Pro	Arg	Ala	Ala
		245			250				255					260	
AGA	CCA	CTG	AAG	AGG	AGG	CAG	CCA	AAG	AAA	ACG	AAC	GAG	CTT	CCG	CAC
Arg	Pro	Leu	Lys	Arg	Arg	Gln	Pro	Lys	Lys	Thr	Asn	Glu	Leu	Pro	His
				265				270					275		
CCC	AAC	AAA	CTC	CCA	GGG	ATC	TTT	GAT	GAT	GGC	CAC	GGT	TCC	CGC	GGC
Pro	Asn	Lys	Leu	Pro	Gly	Ile	Phe	Asp	Asp	Gly	His	Gly	Ser	Arg	Gly
		280					285					290			
AGA	GAG	GTT	TGC	CGC	AGG	CAT	GAG	CTC	TAC	GTC	AGA	TTC	CGT	GAC	CTT
Arg	Glu	Val	Cys	Arg	Arg	His	Glu	Leu	Tyr	Val	Arg	Phe	Arg	Asp	Leu
		295					300					305			
GGC	TGG	CTG	GAC	TGG	GTC	ATC	GCC	CCC	CAG	GGC	TAC	TCT	GCC	TAT	TAC
Gly	Trp	Leu	Asp	Trp	Val	Ile	Ala	Pro	Gln	Gly	Tyr	Ser	Ala	Tyr	Tyr
		310				315				320					
TGT	GAG	GGG	GAG	TGT	GCT	TTC	CCA	CTG	GAC	TCC	TGT	ATG	AAC	GCC	ACC
Cys	Glu	Gly	Glu	Cys	Ala	Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn	Ala	Thr
		325			330				335					340	
AAC	CAT	GCC	ATC	TTG	CAG	TCT	CTG	GTG	CAC	CTG	ATG	AAG	CCA	GAT	GTT
Asn	His	Ala	Ile	Leu	Gln	Ser	Leu	Val	His	Leu	Met	Lys	Pro	Asp	Val
				345				350					355		
GTC	CCC	AAG	GCA	TGC	TGT	GCA	CCC	ACC	AAA	CTG	AGT	GCC	ACC	TCT	GTG
Val	Pro	Lys	Ala	Cys	Cys	Ala	Pro	Thr	Lys	Leu	Ser	Ala	Thr	Ser	Val
		360					365					370			
CTG	TAC	TAT	GAC	AGC	AGC	AAC	AAT	GTC	ATC	CTG	CGT	AAA	CAC	CGT	AAC
Leu	Tyr	Tyr	Asp	Ser	Ser	Asn	Asn	Val	Ile	Leu	Arg	Lys	His	Arg	Asn
		375					380					385			
ATG	GTG	GTC	AAG	GCC	TGT	GGC	TGC	CAC	TGAGGCCCCG	CCCAGCATCC					
Met	Val	Val	Lys	Ala	Cys	Gly	Cys	His							
		390				395									
TGCTTCTACT ACCTTACCAT CTGGCCGGGC CCCTCTCCAG AGGCAGAAAC CCTTCTATGT															

TATCATAGCT CAGACAGGGG CAATGGGAGG CCCTTCACTT CCCCTGGCCA CTTCTGCTA 1433
 5 AAATTCTGGT CTTTCCCACT TCCTCTGTCC TTCATGGGGT TTCGGGGCTA TCACCCCGCC 1493
 CTCTCCATCC TCCTACCCCA AGCATAGACT GAATGCACAC AGCATCCCAG AGCTATGCTA 1553
 ACTGAGAGGT CTGGGGTCAG CACTGAAGGC CCACATGAGG AAGACTGATC CTTGGCCATC 1613
 10 CTCAGCCCAC AATGGCAAAT TCTGGATGGT CTAAGAAGCC CTGGAATTCT AAAC TAGATG 1673
 ATCTGGGCTC TCTGCACCAT TCATTGTGGC AGTTGGGACA TTTT TAGGTA TAACAGACAC 1733
 15 ATACACTTAG ATCAATGCAT CGCTGTACTC CTTGAAATCA GAGCTAGCTT GTTAGAAAAA 1793
 GAATCAGAGC CAGGTATAGC GGTGCATGTC ATTAATCCCA GCGCTAAAGA GACAGAGACA 1853
 GGAGAATCTC TGTGAGTTCA AGGCCACATA GAAAGAGCCT GTCTCGGGAG CAGGAAAAAA 1913
 20 AAAAAAACG GAATTC 1929

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 397 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(D) OTHER INFORMATION: /Product= "mOP2-PP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Met Arg Pro Gly Pro Leu Trp Leu Leu Gly Leu Ala Leu Cys
 1 5 10 15
 Ala Leu Gly Gly Gly His Gly Pro Gly Pro Pro His Thr Cys Pro Gln
 20 25 30
 Arg Arg Leu Gly Ala Arg Asp Arg Asp Met Gln Arg Glu Ile Leu Pro
 35 40 45
 Val Leu Gly Leu Pro Gly Arg Pro Asp Pro Val His Asn Pro Pro Leu
 50 55 60
 Pro Gly Thr Gln Arg Ala Pro Leu Phe Met Leu Asp Leu Tyr His Ala
 65 70 75 80
 Met Thr Asp Asp Asp Asp Gly Gly Pro Pro Gln Ala His Leu Gly Arg
 85 90 95

Ala Asp Leu Val Met Ser Phe Val Asn Met Val Glu Arg Asp Arg Thr
 100 105 110
 5 Leu Gly Tyr Gln Glu Pro His Trp Lys Glu Phe His Phe Asp Leu Thr
 115 120 125
 10 Gln Ile Pro Ala Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile Tyr
 130 135 140
 Lys Glu Pro Ser Thr His Pro Leu Asn Thr Thr Leu His Ile Ser Met
 145 150 155 160
 15 Phe Glu Val Val Gln Glu His Ser Asn Arg Glu Ser Asp Leu Phe Phe
 165 170 175
 Leu Asp Leu Gln Thr Leu Arg Ser Gly Asp Glu Gly Trp Leu Val Leu
 180 185 190
 20 Asp Ile Thr Ala Ala Ser Asp Arg Trp Leu Leu Asn His His Lys Asp
 195 200 205
 Leu Gly Leu Arg Leu Tyr Val Glu Thr Ala Asp Gly His Ser Met Asp
 210 215 220
 25 Pro Gly Leu Ala Gly Leu Leu Gly Arg Gln Ala Pro Arg Ser Arg Gln
 225 230 235 240
 30 Pro Phe Met Val Thr Phe Phe Arg Ala Ser Gln Ser Pro Val Arg Ala
 245 250 255
 Pro Arg Ala Ala Arg Pro Leu Lys Arg Arg Gln Pro Lys Lys Thr Asn
 260 265 270
 35 Glu Leu Pro His Pro Asn Lys Leu Pro Gly Ile Phe Asp Asp Gly His
 275 280 285
 Gly Ser Arg Gly Arg Glu Val Cys Arg Arg His Glu Leu Tyr Val Arg
 290 295 300
 40 Phe Arg Asp Leu Gly Trp Leu Asp Trp Val Ile Ala Pro Gln Gly Tyr
 305 310 315 320
 Ser Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asp Ser Cys
 325 330 335
 45 Met Asn Ala Thr Asn His Ala Ile Leu Gln Ser Leu Val His Leu Met
 340 345 350
 50 Lys Pro Asp Val Val Pro Lys Ala Cys Cys Ala Pro Thr Lys Leu Ser
 355 360 365
 Ala Thr Ser Val Leu Tyr Tyr Asp Ser Ser Asn Asn Val Ile Leu Arg
 370 375 380
 55

Lys His Arg Asn Met Val Val Lys Ala Cys Gly Cys His
 385 390 395

5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 1941 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- 20 (A) ORGANISM: HOMO SAPIENS
 (F) TISSUE TYPE: HIPPOCAMPUS

(ix) FEATURE:

- 25 (A) NAME/KEY: CDS
 (B) LOCATION: 507..1703
 (D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"

/product= "hOP2-PP"
 /note= "hOP2 (CDNA)"

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGAATTCCGG CCACAGTGGC GCCGGCAGAG CAGGAGTGGC TGGAGGAGCT GTGGTTGGAG 60
 35 CAGGAGGTGG CACGGCAGGG CTGGAGGGCT CCCTATGAGT GCGGAGACG GCCCAGGAGG 120
 CGCTGGAGCA ACAGCTCCCA CACCGCACCA AGCGGTGGCT GCAGGAGCTC GCCCATCGCC 180
 CCTGCGCTGC TCGGACCGCG GCCACAGCCG GACTGGCGGG TACGGCGGCG ACAGAGGCAT 240
 40 TGGCCGAGAG TCCAGTCCG CAGAGTAGCC CCGGCCTCGA GCGGTGGCG TCCCGGTCCT 300
 CTCCGTCCAG GAGCCAGGAC AGGTGTCCG CGGCGGGGCT CCAGGGACCG CGCCTGAGGC 360
 45 CGGCTGCCCC CCCGTCCCGC CCCGCCCCGC CGCCCGCCGC CCGCCGAGCC CAGCCTCCTT 420
 GCCGTCGGGG CGTCCCCAGG CCCTGGGTCG GCCGCGGAGC CGATGCGCGC CCGCTGAGCG 480
 CCCCAGCTGA GCGCCCCCGG CCTGCC ATG ACC GCG CTC CCC GGC CCG CTC TGG 533
 50 Met Thr Ala Leu Pro Gly Pro Leu Trp
 1 5
 CTC CTG GGC CTG GCG CTA TGC GCG CTG GGC GGG GGC GGC CCC GGC CTG 581
 Leu Leu Gly Leu Ala Leu Cys Ala Leu Gly Gly Gly Gly Pro Gly Leu
 10 15 20 25
 55 CGA CCC CCG CCC GGC TGT CCC CAG CGA CGT CTG GGC GCG CGC GAC CGG 629

	Arg	Pro	Pro	Pro	Gly	Cys	Pro	Gln	Arg	Arg	Leu	Gly	Ala	Arg	Asp	Arg	
					30					35					40		
5	GAC	GTG	CAG	CGC	GAG	ATC	CTG	GCG	GTG	CTC	GGG	CTG	CCT	GGG	CGG	CCC	677
	Asp	Val	Gln	Arg	Glu	Ile	Leu	Ala	Val	Leu	Gly	Leu	Pro	Gly	Arg	Pro	
				45					50					55			
10	CGG	CCC	CGC	GCG	CCA	CCC	GCC	GCC	TCC	CGG	CTG	CCC	GCG	TCC	GCG	CCG	725
	Arg	Pro	Arg	Ala	Pro	Pro	Ala	Ala	Ser	Arg	Leu	Pro	Ala	Ser	Ala	Pro	
				60				65					70				
15	CTC	TTC	ATG	CTG	GAC	CTG	TAC	CAC	CGC	ATG	GCC	GGC	GAC	GAC	GAC	GAG	773
	Leu	Phe	Met	Leu	Asp	Leu	Tyr	His	Arg	Met	Ala	Gly	Asp	Asp	Asp	Glu	
		75					80					85					
20	GAC	GGC	GCC	GCG	GAG	GCC	CTG	GGC	CGC	GCC	GAC	CTG	GTC	ATG	AGC	TTC	821
	Asp	Gly	Ala	Ala	Glu	Ala	Leu	Gly	Arg	Ala	Asp	Leu	Val	Met	Ser	Phe	
	90					95					100					105	
25	GTT	AAC	ATG	GTG	GAG	CGA	GAC	CGT	GCC	CTG	GGC	CAC	CAG	GAG	CCC	CAT	869
	Val	Asn	Met	Val	Glu	Arg	Asp	Arg	Ala	Leu	Gly	His	Gln	Glu	Pro	His	
					110					115					120		
30	TGG	AAG	GAG	TTC	CGC	TTT	GAC	CTG	ACC	CAG	ATC	CCG	GCT	GGG	GAG	GCG	917
	Trp	Lys	Glu	Phe	Arg	Phe	Asp	Leu	Thr	Gln	Ile	Pro	Ala	Gly	Glu	Ala	
				125					130					135			
35	GTC	ACA	GCT	GCG	GAG	TTC	CGG	ATT	TAC	AAG	GTG	CCC	AGC	ATC	CAC	CTG	965
	Val	Thr	Ala	Ala	Glu	Phe	Arg	Ile	Tyr	Lys	Val	Pro	Ser	Ile	His	Leu	
			140					145					150				
40	CTC	AAC	AGG	ACC	CTC	CAC	GTC	AGC	ATG	TTC	CAG	GTG	GTC	CAG	GAG	CAG	1013
	Leu	Asn	Arg	Thr	Leu	His	Val	Ser	Met	Phe	Gln	Val	Val	Gln	Glu	Gln	
		155					160					165					
45	TCC	AAC	AGG	GAG	TCT	GAC	TTG	TTC	TTT	TTG	GAT	CTT	CAG	ACG	CTC	CGA	1061
	Ser	Asn	Arg	Glu	Ser	Asp	Leu	Phe	Phe	Leu	Asp	Leu	Gln	Thr	Leu	Arg	
	170					175					180					185	
50	GCT	GGA	GAC	GAG	GGC	TGG	CTG	GTG	CTG	GAT	GTC	ACA	GCA	GCC	AGT	GAC	1109
	Ala	Gly	Asp	Glu	Gly	Trp	Leu	Val	Leu	Asp	Val	Thr	Ala	Ala	Ser	Asp	
					190					195					200		
55	TGC	TGG	TTG	CTG	AAG	CGT	CAC	AAG	GAC	CTG	GGA	CTC	CGC	CTC	TAT	GTG	1157
	Cys	Trp	Leu	Leu	Lys	Arg	His	Lys	Asp	Leu	Gly	Leu	Arg	Leu	Tyr	Val	
			205					210					215				
60	GAG	ACT	GAG	GAC	GGG	CAC	AGC	GTG	GAT	CCT	GGC	CTG	GCC	GGC	CTG	CTG	1205
	Glu	Thr	Glu	Asp	Gly	His	Ser	Val	Asp	Pro	Gly	Leu	Ala	Gly	Leu	Leu	
			220					225					230				
65	GGT	CAA	CGG	GCC	CCA	CGC	TCC	CAA	CAG	CCT	TTC	GTG	GTC	ACT	TTC	TTC	1253
	Gly	Gln	Arg	Ala	Pro	Arg	Ser	Gln	Gln	Pro	Phe	Val	Val	Thr	Phe	Phe	

	235	240	245	
5	AGG GCC AGT CCG AGT CCC ATC CGC ACC CCT CGG GCA GTG AGG CCA CTG Arg Ala Ser Pro Ser Pro Ile Arg Thr Pro Arg Ala Val Arg Pro Leu 250 255 260 265	1301		
10	AGG AGG AGG CAG CCG AAG AAA AGC AAC GAG CTG CCG CAG GCC AAC CGA Arg Arg Arg Gln Pro Lys Lys Ser Asn Glu Leu Pro Gln Ala Asn Arg 270 275 280	1349		
15	CTC CCA GGG ATC TTT GAT GAC GTC CAC GGC TCC CAC GGC CGG CAG GTC Leu Pro Gly Ile Phe Asp Asp Val His Gly Ser His Gly Arg Gln Val 285 290 295	1397		
20	TGC CGT CGG CAC GAG CTC TAC GTC AGC TTC CAG GAC CTC GGC TGG CTG Cys Arg Arg His Glu Leu Tyr Val Ser Phe Gln Asp Leu Gly Trp Leu 300 305 310	1445		
25	GAC TGG GTC ATC GCT CCC CAA GGC TAC TCG GCC TAT TAC TGT GAG GGG Asp Trp Val Ile Ala Pro Gln Gly Tyr Ser Ala Tyr Tyr Cys Glu Gly 315 320 325	1493		
30	GAG TGC TCC TTC CCA CTG GAC TCC TGC ATG AAT GCC ACC AAC CAC GCC Glu Cys Ser Phe Pro Leu Asp Ser Cys Met Asn Ala Thr Asn His Ala 330 335 340 345	1541		
35	ATC CTG CAG TCC CTG GTG CAC CTG ATG AAG CCA AAC GCA GTC CCC AAG Ile Leu Gln Ser Leu Val His Leu Met Lys Pro Asn Ala Val Pro Lys 350 355 360	1589		
40	GCG TGC TGT GCA CCC ACC AAG CTG AGC GCC ACC TCT GTG CTC TAC TAT Ala Cys Cys Ala Pro Thr Lys Leu Ser Ala Thr Ser Val Leu Tyr Tyr 365 370 375	1637		
45	GAC AGC AGC AAC AAC GTC ATC CTG CGC AAA GCC CGC AAC ATG GTG GTC Asp Ser Ser Asn Asn Val Ile Leu Arg Lys Ala Arg Asn Met Val Val 380 385 390	1685		
50	AAG GCC TGC GGC TGC CAC TGAGTCAGCC CGCCCAGCCC TACTGCAGCA Lys Ala Cys Gly Cys His 395	1733		
	ATTCACTGGC CGTCGTTTTA CAACGTGTGA CTGGGAAAAC CCTGGCGTTA CCCAACTTAA	1793		
	TCGCCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCTAATA GCGAAGAGGC CCCGCACCGA	1853		
	TCGCCCTTCC CAACAGTTGC GCCCCAGTGA ATGGCGAATG GCAAATTGTA AGCGTTAATA	1913		
	TTTTGTAAAT ATTCGCGTTA AATTTTTT	1941		

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 399 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

5 (D) OTHER INFORMATION: /product= "hOP2-PP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10 Met Thr Ala Leu Pro Gly Pro Leu Trp Leu Leu Gly Leu Ala Leu Cys
 1 5 10 15
 Ala Leu Gly Gly Gly Gly Pro Gly Leu Arg Pro Pro Pro Gly Cys Pro
 20 25 30
 15 Gln Arg Arg Leu Gly Ala Arg Asp Arg Asp Val Gln Arg Glu Ile Leu
 35 40 45
 Ala Val Leu Gly Leu Pro Gly Arg Pro Arg Pro Arg Ala Pro Pro Ala
 20 50 55 60
 Ala Ser Arg Leu Pro Ala Ser Ala Pro Leu Phe Met Leu Asp Leu Tyr
 65 70 75 80
 25 His Arg Met Ala Gly Asp Asp Asp Glu Asp Gly Ala Ala Glu Ala Leu
 85 90 95
 Gly Arg Ala Asp Leu Val Met Ser Phe Val Asn Met Val Glu Arg Asp
 100 105 110
 30 Arg Ala Leu Gly His Gln Glu Pro His Trp Lys Glu Phe Arg Phe Asp
 115 120 125
 Leu Thr Gln Ile Pro Ala Gly Glu Ala Val Thr Ala Ala Glu Phe Arg
 35 130 135 140
 Ile Tyr Lys Val Pro Ser Ile His Leu Leu Asn Arg Thr Leu His Val
 145 150 155 160
 40 Ser Met Phe Gln Val Val Gln Glu Gln Ser Asn Arg Glu Ser Asp Leu
 165 170 175
 Phe Phe Leu Asp Leu Gln Thr Leu Arg Ala Gly Asp Glu Gly Trp Leu
 180 185 190
 45 Val Leu Asp Val Thr Ala Ala Ser Asp Cys Trp Leu Leu Lys Arg His
 195 200 205
 Lys Asp Leu Gly Leu Arg Leu Tyr Val Glu Thr Glu Asp Gly His Ser
 50 210 215 220
 55

Val Asp Pro Gly Leu Ala Gly Leu Leu Gly Gln Arg Ala Pro Arg Ser
 225 230 235 240
 5 Gln Gln Pro Phe Val Val Thr Phe Phe Arg Ala Ser Pro Ser Pro Ile
 245 250 255
 Arg Thr Pro Arg Ala Val Arg Pro Leu Arg Arg Arg Gln Pro Lys Lys
 260 265 270
 10 Ser Asn Glu Leu Pro Gln Ala Asn Arg Leu Pro Gly Ile Phe Asp Asp
 275 280 285
 Val His Gly Ser His Gly Arg Gln Val Cys Arg Arg His Glu Leu Tyr
 290 295 300
 15 Val Ser Phe Gln Asp Leu Gly Trp Leu Asp Trp Val Ile Ala Pro Gln
 305 310 315 320
 Gly Tyr Ser Ala Tyr Tyr Cys Glu Gly Glu Cys Ser Phe Pro Leu Asp
 325 330 335
 20 Ser Cys Met Asn Ala Thr Asn His Ala Ile Leu Gln Ser Leu Val His
 340 345 350
 Leu Met Lys Pro Asn Ala Val Pro Lys Ala Cys Cys Ala Pro Thr Lys
 355 360 365
 25 Leu Ser Ala Thr Ser Val Leu Tyr Tyr Asp Ser Ser Asn Asn Val Ile
 370 375 380
 Leu Arg Lys Ala Arg Asn Met Val Val Lys Ala Cys Gly Cys His
 385 390 395
 30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: Protein
 (B) LOCATION: 1..102
 (D) OTHER INFORMATION: /label= OPX

/note= "WHEREIN EACH XAA IS INDEPENDENTLY
 SELECTED FROM A GROUP OF ONE OR MORE
 SPECIFIED AMINO ACIDS AS DEFINED IN THE
 SPECIFICATION

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) **FEATURE:**

- (A) NAME/KEY: Protein
(B) LOCATION: 1..97
(D) OTHER INFORMATION: /label= OPX-7C

/note= "WHEREIN EACH XAA INDEPENDENTLY INDICATES ONE OF THE 20 NATURALLY-OCCURRING L-ISOMER, A-AMINO ACIDS, OR A DERIVATIVE THEREOF."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa**
 1 5 10 15
Xaa **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Cys** **Xaa** **Xaa** **Xaa** **Cys** **Xaa** **Xaa** **Xaa**
 20 25 30
Xaa **Xaa** **Xaa** **Cys** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa**
 35 40 45
Xaa **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Xaa** **Cys** **Cys** **Xaa** **Xaa**
 50 55 60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 65 70 75 80

5 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys
 85 90 95

Xaa

10 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 102 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

20 (ix) FEATURE:

(A) NAME/KEY: Protein
 (B) LOCATION: 1..102
 (D) OTHER INFORMATION: /label= OPX-8C

25 /note= "WHEREIN EACH XAA INDEPENDENTLY
 INDICATES ONE OF THE 20
 NATURALLY-OCCURRING L-ISOMER A-AMINO
 ACIDS, OR A DERIVATIVE THEREOF."

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

35 Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1 5 10 15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa
 20 25 30

40 Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 50 55 60

45 Xaa Cys Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 65 70 75 80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 85 90 95

50 Xaa Xaa Cys Xaa Cys Xaa
 100

Claims

- 55
1. A polypeptide chain comprising an amino acid sequence described by residues 303-399 of Seq. ID No. 5.
 2. The polypeptide chain of claim 1 comprising an amino acid sequence described by

(a) residues 298-399 of Seq. ID No. 5; or

(b) residues 267-399 of Seq. ID No. 5; or

(c) residues 264-399 of Seq. ID No. 5; or

(d) residues 240-399 of Seq. ID No. 5; or

(e) residues 1-399 of Seq. ID No. 5.

3. A polypeptide chain comprising an amino acid sequence described by residues of 301-397 of Seq. ID No. 3.

4. The polypeptide chain of claim 3 comprising an amino acid sequence described by (a) residues 296-397 of Seq. ID No.3; or

(b) residues 259-397 of Seq. ID No. 3; or

(c) residues 1-397 of Seq. ID No. 3.

5. A polypeptide chain useful as a subunit of a dimeric osteogenic protein comprising a pair of disulphide-bonded polypeptide chains, said polypeptide chain comprising an amino acid sequence which either:

(a) shares the conserved six cysteine skeleton depicted in Fig. 2.1 to 2.3 from Leu (position 43) to His (position 139) of hOP1 and further comprising an additional cysteine residue in said six cysteine skeleton, or

(b) shares the conserved seven cysteine skeleton depicted in Fig. 2.1 to 2.3 from Cys (position 38) to His (position 139) of hOP1 and further comprises an additional cysteine residue therein,

such that the dimeric osteogenic protein comprising said polypeptide chain has a conformation capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix.

6. The polypeptide chain of claim 5 wherein said amino acid sequence comprises:

(a) residues 261-399 of Seq. ID No. 5; or

(b) residues 301-397 of Seq. ID No. 3; or

(c) residues 259-397 of Seq. ID No. 3; or

(d) residues 298-399 of Seq. ID No. 5.

7. A dimeric osteogenic protein capable of inducing endochondral bone formation in a mammal when implanted in said mammal in association with a matrix, said protein comprising a pair of disulphide-bonded polypeptide chains constituting a dimeric species, wherein each of said polypeptide chains is the polypeptide chain of claim 5.

8. The polypeptide chain of any one of claims 1 to 6 produced by expression of recombinant DNA in host cell, e.g. a prokaryotic host cell or a mammalian host cell.

9. The polypeptide chain of claim 8 wherein said host cell is selected from *E. coli*, CHO, COS, BSC, *Saccharomyces* or myeloma host cells.

10. The polypeptide chain of any one of claims 1 to 6 that is glycosylated.

11. A nucleic acid encoding the polypeptide chain of any one of claims 1 to 6.

12. A polypeptide chain encoded by a gene comprising:

(a) the DNA sequence described by Seq. ID No. 3;

(b) the DNA sequence described by Seq. ID No. 5;

(c) a DNA sequence hybridizable under stringent hybridization conditions with nucleotides 467-771 of Seq. ID No. 3 and encoding a polypeptide chain comprising an amino acid sequence which either: (a) shares the conserved six cysteine skeleton depicted in Fig. 2.1 to 2.3 from Leu (position 43) to His (position 139) of hOP1 and further comprises an additional cysteine residue in said six cysteine skeleton, or (b) shares the conserved

seven cysteine skeleton depicted in Fig. 2.1 to 2.3 from Cys (position 38) to His (position 139) of hOP1 and further comprises an additional cysteine residue therein.

such that the dimeric osteogenic protein comprising said polypeptide chain has a conformation capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix.

13. An isolated antibody, e.g. a polyclonal or monoclonal antibody, having binding specificity only for a polypeptide or protein as defined in any one of claims 1-10 and 12.

14. The antibody of claim 13 having binding specificity for a protein comprising an amino acid sequence described by:

- (a) residues 303-399 of Seq. ID No. 5;
- (b) residues 297-399 of Seq. ID No. 5;
- (c) residues 264-399 of Seq. ID No. 5;
- (d) residues 1-399 of Seq. ID No. 5;
- (e) residues 18-257 of Seq. ID No. 5;
- (f) residues 301-397 of Seq. ID No. 3;
- (g) residues 296-397 of Seq. ID No. 3;
- (h) residues 259-397 of Seq. ID No. 3;
- (i) residues 1-397 of Seq. ID No. 3; or
- (j) residues 18-259 of Seq. ID No. 3.

15. A DNA molecule encoding the protein of claim 12.

16. The DNA molecule of claim 15 wherein said molecule comprises:

- (a) the DNA sequence described by Seq. ID No. 3;
- (b) the DNA sequence described by Seq. ID No. 5; or
- (c) the DNA sequence hybridizable with nucleotides 467-771 of Seq. ID No. 3.

17. A protein comprising a pair of polypeptide chains and competent to induce cartilage or bone formation in a mammal, each of said polypeptide chains comprising the amino acid sequence defined by OPX (Seq. ID No. 7), wherein Xaa at residue 41 is Cys.

18. The protein of claim 7 or 12 in combination with a biocompatible, *in vivo* biodegradable carrier, said carrier acting as a slow release delivery system.

19. An implantable osteogenic device comprising the polypeptide chain of any one of claims 1-6, 8-10 and 12, or the protein of any one of claims 7, 17 and 18.

20. An osteogenic device for implantation in a mammal, the device comprising:

- (a) a biocompatible, in vivo biodegradable matrix having pores of a dimension sufficient to permit the influx, differentiation and proliferation of migratory progenitor cells from the body of said mammal; and
- (b) the polypeptide chain of any one of claims 1-6, 8-10 and 12, or the protein of any one of claims 7, 17 and 18.

21. The device of claim 20 wherein said matrix comprises allogenic or xenogenic bone.

22. The device of any one of claims 19-21 wherein said matrix comprises demineralized, delipidated, Type I insoluble bone collagen particles, depleted in noncollagenous protein, and treated to increase the intraparticle intrusion volume and surface area, for example with:

- (a) a collagen fibril modifying agent; or
- (b) a protease (e.g. trypsin); or
- (c) a solvent (e.g. dichloromethane, trichloroacetic acid and acetonitrile); or
- (d) an acid (e.g. trifluoroacetic acid or hydrogen fluoride); or
- (e) a hot aqueous medium having a temperature within the range of 37 degrees centigrade to 75 degrees centigrade.

23. A process for producing the device of claim 22 comprising the step of treating collagen to increase the intraparticle intrusion volume and surface area.
24. The polypeptide chain of any one of claims 1-6, 8-10 and 12, or the protein of any one of claims 7, 17 and 18 in association with a matrix, wherein the matrix comprises:
- (a) allogenic bone (for example, particulate, demineralized and guanidine extracted allogenic bone); or
 - (b) xenogenic bone (for example particulate, protein extracted, demineralized, xenogenic bone); or
 - (c) particulate, protein extracted, demineralized, xenogenic bone treated with a protease or a fibril modifying agent to increase the intraparticle intrusion volume and surface area, e.g. a solvent; or
 - (d) materials selected from collagen, homopolymers or copolymers of glycolic acid and lactic acid, hydroxyapatite and calcium phosphate (e.g. tricalcium phosphate) ; or
 - (e) a shape-retaining solid of loosely adhered particulate material, for example collagen; or
 - (f) a molded, porous solid; or
 - (g) masticated tissue (e.g. muscle).
25. An active heterodimeric osteogenic protein comprising the polypeptide chain of any one of claims 1-6, 8-10 and 12, or the protein of any one of claims 7, 17 and 18.
26. The heterodimeric protein of claim 25 wherein the heterodimers are joined by disulphide bonds or otherwise associated.
27. A process for producing the heterodimeric protein of claim 25 or 26 comprising the step of oxidising and refolding two or more polypeptide chains.

Patentansprüche

1. Polypeptidkette, die eine Aminosäure-Sequenz enthält, die die Gruppen 303-399 der Sequenz ID Nr. 5 enthält.
2. Polypeptidkette nach Anspruch 1, die eine Aminosäure-Sequenz enthält, die die Gruppen
- a) 298-399 der Sequenz ID Nr. 5 oder
 - b) 267-399 der Sequenz ID Nr. 5 oder
 - c) 264-399 der Sequenz ID Nr. 5 oder
 - d) 240-399 der Sequenz ID Nr. 5 oder
 - e) 1-399 der Sequenz ID Nr. 5
- enthält.
3. Polypeptidkette, die eine Aminosäure-Sequenz enthält, die die Gruppen 301-397 der Sequenz ID Nr. 3 enthält.
4. Polypeptidkette nach Anspruch 3, die eine Aminosäure-Sequenz enthält, die die Gruppen
- a) 296-397 der Sequenz ID Nr. 3 oder
 - b) 259-397 der Sequenz ID Nr. 3 oder
 - c) 1-397 der Sequenz ID Nr. 3
- enthält.
5. Polypeptidkette, verwendbar als Untereinheit für ein dimeres osteogenes Protein, das ein Paar disulfidgebundene Polypeptidketten, wobei die Polypeptidkette eine Aminosäure-Sequenz enthält, die entweder
- a) das erhaltene sechs Cystein-Gerüst von menschlichem OP1, dargestellt in Fig. 2.1 bis 2.3 von Leu (Position 43) bis His (Position 139), umschließt und weiterhin eine zusätzliche Cystein-Gruppe in diesem sechs Cystein-Gerüst enthält oder
 - b) das erhaltene sieben Cystein-Gerüst von menschlichem OP1, dargestellt in Fig. 2.1 bis 2.3 von Cys (Position

38) bis His (Position 139), umschließt und weiterhin eine zusätzliche Cystein-Gruppe darin enthält,

so daß das diese Polypeptidkette enthaltende, dimere osteogene Protein einen Aufbau hat, der eine endochondrale Knochenbildung zu induzieren in der Lage ist, wenn er in Verbindung mit einer Matrix einem Säuger implantiert wird.

6. Polypeptidkette nach Anspruch 5, wobei die beschriebene Aminosäure-Sequenz die Gruppen

- a) 261-399 der Sequenz ID Nr. 5 oder
- b) 301-397 der Sequenz ID Nr. 3 oder
- c) 259-397 der Sequenz ID Nr. 3 oder
- d) 298-299 der Sequenz ID Nr. 5

enthält.

7. Dimeres osteogenes Protein, das eine endochondrale Knochenbildung in einem Säuger zu induzieren in der Lage ist, wenn es diesem Säuger in Verbindung mit einer Matrix implantiert wird, und ein Paar disulfidgebundene Polypeptidketten, die eine dimere Spezies bilden, enthält, wobei jede dieser Polypeptidketten die Polypeptidkette nach Anspruch 5 ist.

8. Polypeptidkette nach einem der Ansprüche 1 bis 6, hergestellt durch die Produktion neuer Gene rekombinanter DNS in einer Wirtszelle, z. B. einer prokaryontischen Wirtszelle oder einer Wirtszelle eines Säugers.

9. Polypeptidkette nach Anspruch 8, wobei die Wirtszelle aus Wirtszellen von E. coli, CHO, COS, BSC, Saccharomyces oder myeloma ausgewählt ist.

10. Polypeptidkette nach einem der Ansprüche 1 bis 6, die glycosyliert ist.

11. Nukleinsäure, die die Polypeptidkette nach einem der Ansprüche 1 bis 6 codiert.

12. Polypeptidkette, die mit einem Gen codiert ist, das

- a) die DNS-Sequenz der Sequenz ID Nr. 3;
- b) die DNS-Sequenz der Sequenz ID Nr. 5;
- c) eine unter zwingend hybridisierenden Bedingungen hybridisierbare DNS-Sequenz mit den Nucleotiden 467-771 der Sequenz ID Nr. 3 enthält und eine Polypeptidkette codiert, die eine Aminosäure-Sequenz enthält, die entweder

a) das erhaltene sechs Cystein-Gerüst von menschlichem OP1, dargestellt in Fig. 2.1 bis 2.3 von Leu (Position 43) bis His (Position 139), umschließt und weiterhin eine zusätzliche Cystein-Gruppe in diesem sechs Cystein-Gerüst enthält oder

b) das erhaltene sieben Cystein-Gerüst von menschlichem OP1, dargestellt in Fig. 2.1 bis 2.3 von Cys (Position 38) bis His (Position 139), umfaßt und weiterhin eine zusätzliche Cystein-Gruppe darin enthält,

so daß das dimere osteogene Protein, welches diese Polypeptidkette enthält, einen Aufbau hat, der eine endochondrale Knochenbildung zu induzieren in der Lage ist, wenn er in Verbindung mit einer Matrix einem Säuger implantiert wird.

13. Isolierter Antikörper, z. B. polyklonaler oder monoklonaler Antikörper, der eine Bindungsspezifität nur für ein Polypeptid oder ein Protein nach einem der Ansprüche 1 bis 10 und 12 aufweist.

14. Antikörper nach Anspruch 13, der eine Bindungsspezifität für ein Protein mit einer Aminosäure-Sequenz aufweist, die die Gruppen

- a) 303-399 der Sequenz ID Nr. 5;
- b) 297-399 der Sequenz ID Nr. 5;
- c) 264-399 der Sequenz ID Nr. 5;

- d) 1-399 der Sequenz ID Nr. 5;
- e) 18-257 der Sequenz ID Nr. 5;
- f) 301-397 der Sequenz ID Nr. 3;
- g) 296-397 der Sequenz ID Nr. 3;
- h) 259-397 der Sequenz ID Nr. 3;
- i) 1-397 der Sequenz ID Nr. 3 oder
- j) 18-259 der Sequenz ID Nr. 3

enthält.

15. DNS-Molekül, das das Protein nach Anspruch 12 codiert.

16. DNS-Molekül nach Anspruch 15, das

- a) die DNS-Sequenz der Sequenz ID Nr. 3;
- b) die DNS-Sequenz der Sequenz ID Nr. 5 oder
- c) die hybridisierbare DNS-Sequenz mit den Nukleotiden 467-771 der Sequenz ID Nr. 3

enthält.

17. Protein, das für die Knorpel- oder Knochenbildung bei einem Säuger maßgeblich ist und ein Paar Polypeptidketten enthält, die beide eine durch OPX (Sequenz ID Nr. 7) definierte Aminosäure-Sequenz enthalten, wobei Xaa in der Gruppe 41 Cys ist.

18. Protein nach Anspruch 7 oder 12 in Kombination mit einem biokompatiblen, in vivo biologisch abbaubaren Träger, der als langsam freisetzendes Versorgungssystem wirkt.

19. Implantierbares osteogenes Mittel, das die Polypeptidkette nach einem der Ansprüche 1 bis 6, 8 bis 10 und 12 oder das Protein nach einem der Ansprüche 7, 17 und 18 enthält.

20. Osteogenes Mittel für die Implantation in einen Säuger, das

a) eine biokompaktible, *in vivo* biologisch abbaubare Matrix mit einer ausreichenden Porengröße für die Zufuhr, die Differenzierung und das Wachstum von umgesiedelten Vorläufer-Zellen aus dem Körper dieses Säugers und

b) die Polypeptidkette nach einem der Ansprüche 1 bis 6, 8 bis 10 und 12, oder das Protein nach einem der Ansprüche 7, 17 und 18

enthält.

21. Mittel nach Anspruch 20, wobei die Matrix allogenes oder xenogenes Knochenmaterial enthält.

22. Mittel nach einem der Ansprüche 19 bis 21, wobei die Matrix demineralisierte, delipidisierte, Typ I unlösliche Knochen-Kollagenpartikeln enthält, deren nicht kollagene Proteine abgereichert sind, und die zur Vergrößerung des intrapartikulären Eindringvolumens und der Oberfläche z. B. mit:

- a) einem Kollagenfibrillen modifizierenden Mittel oder
- b) einer Protease (z. B. Trypsin) oder
- c) einem Lösungsmittel (z. B. Dichlormethan, Trichloressigsäure und Acetonitril) oder
- d) einer Säure (z. B. Trifluoressigsäure oder Fluorwasserstoff) oder
- e) einem heißen wäßrigen Medium mit einer Temperatur im Bereich von 37°C bis 75°C

behandelt sind.

23. Verfahren zur Herstellung des Mittels nach Anspruch 22 mit dem Schritt der Kollagenbehandlung zur Vergrößerung des intrapartikulären Eindringvolumens und der Oberfläche.

24. Polypeptidkette nach einem der Ansprüche 1 bis 6, 8 bis 10 und 12, oder Protein nach einem der Ansprüche 7, 17 und 18 in Verbindung mit einer Matrix, die

- a) allogenenes Knochenmaterial (z. B. partikelförmiges, demineralisiertes und Guanidin extrahiertes allogenenes Knochenmaterial) oder
- b) xenogenes Knochenmaterial (z. B. partikelförmiges, demineralisiertes, Protein extrahiertes xenogenes Knochenmaterial) oder
- c) partikelförmiges, demineralisiertes, Protein extrahiertes xenogenes Knochenmaterial, das mit einer Protease oder einem Fibrillen modifizierenden Mittel zur Vergrößerung des intrapartikulären Eindringvolumens und der Oberfläche, z. B. einem Lösungsmittel, behandelt ist oder
- d) aus Kollagen ausgewähltes Material, Homo- oder - Copolymeren von Hydroxyessigsäure und Milchsäure, Hydroxyapatit und Calciumphosphat (z. B. Tricalciumphosphat) oder
- e) ein formstabiles, festes oder lose zusammenhängendes, partikelförmiges Material, z. B. Kollagen oder
- f) einen geformten, porösen Feststoff oder
- g) zerkleinertes Gewebe (z. B. Muskelgewebe)

enthält.

25. Aktives heterodimeres osteogenes Protein, das die Polypeptidkette nach einem der Ansprüche 1 bis 6, 8 bis 10 und 12, oder das Protein nach einem der Ansprüche 7, 17 und 18 enthält.

26. Heterodimeres Protein nach Anspruch 25, wobei die Heterodimere über Disulfidbindungen oder anderweitig gebunden sind.

27. Verfahren zur Herstellung des heterodimeren Proteins nach Anspruch 25 oder 26 durch Oxidieren und Falten von zwei oder mehr Polypeptidketten.

Revendications

1. Chaîne polypeptidique comprenant une séquence d'acides aminés décrite par les résidus 303-399 de Seq. ID No. 5.

2. Chaîne polypeptidique selon la revendication 1, comprenant une séquence d'acides aminés décrite par:

- (a) les résidus 298-399 de Seq. ID No. 5; ou
- (b) les résidus 267-399 de Seq. ID No. 5; ou
- (c) les résidus 264-399 de Seq. ID No. 5; ou
- (d) les résidus 240-399 de Seq. ID No. 5; ou
- (e) les résidus 1-399 de Seq. ID No. 5.

3. Chaîne polypeptidique comprenant une séquence d'acides aminés décrite par les résidus 301-397 de Seq. ID No. 3.

4. Chaîne polypeptidique selon la revendication 3, comprenant une séquence d'acides aminés décrite par:

- (a) les résidus 296-397 de Seq. ID No. 3; ou
- (b) les résidus 259-397 de Seq. ID No. 3; ou
- (c) les résidus 1-397 de Seq. ID No. 3.

5. Chaîne polypeptidique utile comme sous-unité d'une protéine ostéogène dimère comprenant une paire de chaînes polypeptidiques à liaisons disulfures, lesdites chaînes polypeptidiques comprenant une séquence d'acides aminés qui, soit

- (a) partage le squelette conservé de six résidus cystéine décrit dans les figures 2.1 à 2.3 depuis le résidu Leu (position 43) jusqu'au résidu His (position 139) de hOP1, et comprend en outre un résidu cystéine supplémentaire dans ledit squelette de six résidus cystéine, soit
- (b) partage le squelette conservé de sept résidus cystéine décrits dans les figures 2.1 à 2.3 depuis le résidu

Cys (position 38) jusqu'au résidu His (position 139) de hOP1, et comprend en outre un résidu cystéine supplémentaire dans ledit squelette,

de telle sorte que la protéine ostéogène dimère comprenant ladite chaîne polypeptidique possède une conformation capable d'induire une formation d'os cartilagineux lorsqu'elle est implantée dans un mammifère en association avec une matrice.

6. Chaîne polypeptidique selon la revendication 5, dans laquelle ladite séquence d'acides aminés comprend:

- (a) les résidus 261-399 de Seq. ID No. 5; ou
- (b) les résidus 301-397 de Seq. ID No. 3; ou
- (c) les résidus 259-397 de Seq. ID No. 3; ou
- (d) les résidus 298-399 de Seq. ID No. 5.

7. Protéine ostéogène dimère capable d'induire une formation d'os cartilagineux dans un mammifère lorsqu'elle est implantée dans ledit mammifère en association avec une matrice, ladite protéine comprenant une paire de chaînes polypeptidiques à liaisons disulfures Constituant une espèce dimère, dans laquelle chacune desdites chaînes polypeptidiques est la chaîne polypeptidique selon la revendication 5.

8. Chaîne polypeptidique selon l'une quelconque des revendications 1 à 6, obtenue par expression d'ADN recombinant dans une cellule hôte, par exemple une cellule hôte procaryote ou une cellule hôte mammalienne.

9. Chaîne polypeptidique selon la revendication 8, dans laquelle ladite cellule hôte est choisie parmi des cellules hôtes *E. coli*, CHO, COS, BSC, *Saccharomyces* ou de myélomes.

10. Chaîne polypeptidique selon l'une quelconque des revendications 1 à 6, à l'état glycosylé.

11. Acide nucléique encodant la chaîne polypeptidique selon l'une quelconque des revendications 1 à 6.

12. Chaîne polypeptidique encodée par un gène comprenant:

- (a) la séquence d'ADN décrite par Seq. ID No. 3;
- (b) la séquence d'ADN décrite par Seq. ID No. 5;
- (c) une séquence d'ADN apte à s'hybrider dans des conditions d'hybridation sévères avec les nucléotides 467-771 de Seq. ID No. 3 et encodant une chaîne polypeptidique comprenant une séquence d'acides aminés qui, soit:

- a) partage le squelette conservé de six résidus cystéine décrits dans les figures 2.1 à 2.3 depuis le résidu Leu (position 43) jusqu'au résidu His (position 139) de hOP1, et comprend en outre un résidu cystéine; supplémentaire dans ledit squelette de six résidus cystéine;
- b) soit partage le squelette conservé de sept résidus cystéine décrits dans les figures 2.1 à 2.3 depuis le résidu Cys (position 38) jusqu'au résidu His (position 139) de hOP1, et comprend en outre un résidu cystéine supplémentaire dans ledit squelette,

de telle sorte que la protéine ostéogène dimère comprenant ladite chaîne polypeptidique possède une conformation capable d'induire une formation d'os cartilagineux lorsqu'elle est implantée dans un mammifère en association avec une matrice.

13. Anticorps isolé, par exemple anticorps polyclonal ou monoclonal possédant une spécificité de liaison uniquement pour un polypeptide ou pour une protéine tels que définis dans l'une quelconque des revendications 1-10 et 12.

14. Anticorps selon la revendication 13, possédant une spécificité de liaison pour une protéine comprenant une séquence d'acides aminés décrite par:

- (a) les résidus 303-399 de Seq. ID No. 5;
- (b) les résidus 297-399 de Seq. ID No. 5;
- (c) les résidus 264-399 de Seq. ID No. 5;
- (d) les résidus 1-399 de Seq. ID No. 5;

- (e) les résidus 18-257 de Seq. ID No. 5;
- (f) les résidus 301-397 de Seq. ID No. 3;
- (g) les résidus 296-397 de Seq. ID No. 3;
- (h) les résidus 259-397 de Seq. ID No. 3;
- (i) les résidus 1-397 de Seq. ID No. 3; ou
- (j) les résidus 18-259 de Seq. ID No. 3.

15. Molécule d'ADN encodant la protéine selon la revendication 12.

16. Molécule d'ADN selon la revendication 15, dans laquelle ladite molécule comprend:

- (a) la séquence d'ADN décrite par Seq. ID No. 3;
- (b) la séquence d'ADN décrite par Seq. ID No. 5; ou
- (c) la séquence d'ADN apte à s'hybrider avec les nucléotides 467-771 de Seq. ID No. 3.

17. Protéine comprenant une paire de chaînes polypeptidiques et compétente pour induire une formation d'os ou de cartilage dans un mammifère, chacune desdites chaînes polypeptidiques comprenant la séquence d'acides aminés définie par OPX (Seq. ID No. 7) dans laquelle Xaa au résidu 41 représente un résidu Cys.

18. Protéine selon la revendication 7 ou 12, en combinaison avec un support biocompatible biodégradable in vivo, ledit support faisant office de système à libération lente.

19. Dispositif ostéogène implantable comprenant la chaîne polypeptidique selon l'une quelconque des revendications 1 à 6, 8-10 et 12, ou la protéine selon l'une quelconque des revendications 7, 17 et 18.

20. Dispositif ostéogène destiné à une implantation dans un mammifère, le dispositif comprenant:

- (a) une matrice biocompatible biodégradable in vivo possédant des pores de dimensions suffisantes pour permettre le flux entrant, la différenciation et la prolifération de cellules souches migratoires à partir du corps dudit mammifère; et
- (b) la chaîne polypeptidique selon, l'une quelconque des revendications 1 à 6, 8 à 10 et 12, ou la protéine selon l'une quelconque des revendications 7, 17 et 18.

21. Dispositif selon la revendication 20, dans lequel ladite matrice comprend de l'os allogénique ou xénogène.

22. Dispositif selon l'une quelconque des revendications 19 à 21, dans lequel ladite matrice comprend des particules de collagène osseux insolubles de type I, déminéralisées, délipidées, appauvries en protéines non collagéniques et traitées pour augmenter le volume d'intrusion intraparticulaire et l'aire de surface, par exemple avec:

- (a) un modificateur des fibrilles du collagène;
- (b) une protéase (par exemple la trypsine); ou
- (c) un solvant (par exemple le dichlorométhane, l'acide trichloracétique et l'acétonitrile); ou
- (d) un acide (par exemple l'acide trifluoracétique ou l'acide fluorhydrique); ou
- (e) un milieu aqueux chaud dont la température se situe dans le domaine de 37 degrés centigrades à 75 degrés centigrades.

23. Procédé pour préparer le dispositif selon la revendication 22, comprenant l'étape consistant à traiter du collagène pour augmenter le volume d'intrusion intraparticulaire et l'aire de surface.

24. Chaîne polypeptidique selon l'une quelconque des revendications 1 à 6, 8 à 10 et 12, ou protéine selon l'une quelconque des revendications 7, 17 et 18, en association avec une matrice, la matrice comprenant:

- (a) de l'os allogénique (par exemple de l'os allogénique particulaire, déminéralisé et extrait dans de la guanidine); ou
- (b) de l'os xénogène (par exemple de l'os xénogène, particulaire déminéralisé qui est extrait dans une protéine); ou
- (c) de l'os xénogène particulaire, déminéralisé, extrait dans une protéine, traité avec une protéase ou avec un modificateur des fibrilles pour augmenter le volume d'intrusion intraparticulaire et l'aire de surface, par

exemple un solvant; ou

(d) des matières choisies parmi le collagène, des homopolymères ou des copolymères de l'acide glycolique et de l'acide lactique, l'hydroxyapatite et le phosphate de calcium, par exemple le phosphate tricalcique); ou

(e) un produit solide indéformable d'une matière particulaire à adhérence lâche, par exemple le collagène; ou

(f) un produit solide poreux moulé; ou

(g) un tissu masticateur (par exemple un muscle).

25. Protéine active ostéogène hétérodimère comprenant la chaîne polypeptidique selon l'une quelconque des revendications 1 à 6, 8 à 10 et 12, ou la protéine selon l'une quelconque des revendications 7, 17 et 18.

26. Protéine hétérodimère selon la revendication 25, dans laquelle les hétérodimères sont joints par des liaisons disulfures ou associés d'une autre manière.

27. Procédé pour préparer la protéine hétérodimère selon la revendication 25 ou 26, comprenant l'étape consistant à soumettre à une oxydation et à un repliage deux chaînes polypeptidiques ou plus.

hOP2	Ala	Val	Arg	Pro	Leu	Arg	Arg	Arg
mOP2	...	Ala	Lys
					5			
hOP2	Gln	Pro	Lys	Lys	Ser	Asn	Glu	Leu
mOP2	Thr
		10				15		
hOP2	Pro	Gln	Ala	Asn	Arg	Leu	Pro	Gly
mOP2	...	His	Pro	...	Lys
				20				
hOP2	Ile	Phe	Asp	Asp	Val	His	Gly	Ser
mOP2	Gly
	25					30		
hOP2	His	Gly	Arg	Gln	Val	Cys	Arg	Arg
mOP2	Arg	Glu
			35					40
hOP2	His	Glu	Leu	Tyr	Val	Ser	Phe	Gln
mOP2	Arg	...	Arg
					45			
hOP2	Asp	Leu	Gly	Trp	Leu	Asp	Trp	Val
mOP2
		50					55	
hOP2	Ile	Ala	Pro	Gln	Gly	Tyr	Ser	Ala
mOP2
				60				
hOP2	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ser
mOP2	Ala
	65					70		

Fig. 1.1

hOP2	Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn
mOP2
			75					80
hOP2	Ala	Thr	Asn	His	Ala	Ile	Leu	Gln
mOP2
				85				
hOP2	Ser	Leu	Val	His	Leu	Met	Lys	Pro
mOP2
		90					95	
hOP2	Asn	Ala	Val	Pro	Lys	Ala	Cys	Cys
mOP2	Asp	Val
			100					
hOP2	Ala	Pro	Thr	Lys	Leu	Ser	Ala	Thr
mOP2
	105					110		
hOP2	Ser	Val	Leu	Tyr	Tyr	Asp	Ser	Ser
mOP2
			115					120
hOP2	Asn	Asn	Val	Ile	Leu	Arg	Lys	Ala
mOP2	His
				125				
hOP2	Arg	Asn	Met	Val	Val	Lys	Ala	Cys
mOP2
		130					135	
hOP2	Gly	Cys	His					
mOP2					

Fig. 1.2

hOP1	Ser	Thr	Gly	Ser	Lys	Gln	Arg	Ser	Gln
mOP1	Gly
hOP2	Ala	Val	Arg	Pro	Leu	Arg	...	Arg	...
mOP2	Ala	Ala	Arg	Pro	Leu	Lys	...	Arg	...
	1				5				
hOP1	Asn	Arg	Ser	Lys	Thr	Pro	Lys	Asn	Gln
mOP1
hOP2	Pro	Lys	Lys	Ser	Asn	Glu	Leu	Pro	Gln
mOP2	Pro	Lys	Lys	Thr	Asn	Glu	Leu	Pro	His
	10					15			
hOP1	Glu	Ala	Leu	Arg	Met	Ala	Asn	Val	Ala
mOP1	Ser
hOP2	Ala	Asn	Arg	Leu	Pro	Gly	Ile	Phe	Asp
mOP2	Pro	Asn	Lys	Leu	Pro	Gly	Ile	Phe	Asp
		20					25		
hOP1	Glu	Asn	Ser	Ser	Ser	Asp	Gln	Arg	Gln
mOP1
hOP2	Asp	Val	His	Gly	...	His	Gly
mOP2	Asp	Gly	His	Gly	...	Arg	Gly	...	Glu
			30					35	
hOP1	Ala	Cys	Lys	Lys	His	Glu	Leu	Tyr	Val
mOP1
hOP2	Val	...	Arg	Arg
mOP2	Val	...	Arg	Arg
				40					45

Fig. 2.1

hOP1	Ser	Phe	Arg	Asp	Leu	Gly	Trp	Gln	Asp
mOP1
hOP2	Gln	Leu	...
mOP2	Arg	Leu	...
					10				
hOP1	Trp	Ile	Ile	Ala	Pro	Glu	Gly	Tyr	Ala
mOP1
hOP2	...	Val	Gln	Ser
mOP2	...	Val	Gln	Ser
	55					60			
hOP1	Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala
mOP1
hOP2	Ser
mOP2
		65					70		
hOP1	Phe	Pro	Leu	Asn	Ser	Tyr	Met	Asn	Ala
mOP1
hOP2	Asp	...	Cys
mOP2	Asp	...	Cys
			75					80	
hOP1	Thr	Asn	His	Ala	Ile	Val	Gln	Thr	Leu
mOP1
hOP2	Leu	...	Ser	...
mOP2	Leu	...	Ser	...
				85					90

Fig. 2.2

hOP1	Val	His	Phe	Ile	Asn	Pro	Glu	Thr	Val
mOP1	Asp
hOP2	Leu	Met	Lys	...	Asn	Ala	...
mOP2	Leu	Met	Lys	...	Asp	Val	...

95

hOP1	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Gln
mOP1
hOP2	Ala	Lys
mOP2	Ala	Lys

100

105

hOP1	Leu	Asn	Ala	Ile	Ser	Val	Leu	Tyr	Phe
mOP1
hOP2	...	Ser	...	Thr	Tyr
mOP2	...	Ser	...	Thr	Tyr

110

115

hOP1	Asp	Asp	Ser	Ser	Asn	Val	Ile	Leu	Lys
mOP1	Asp
hOP2	...	Ser	...	Asn	Arg
mOP2	...	Ser	...	Asn	Arg

120

125

hOP1	Lys	Tyr	Arg	Asn	Met	Val	Val	Arg
mOP1
hOP2	...	Ala	Lys
mOP2	...	His	Lys

130

hOP1	Ala	Cys	Gly	Cys	His
mOP1
hOP2
mOP2

135

Fig. 2.3